The BBX gene CmBBX22 negatively regulates drought stress tolerance in chrysanthemum

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Abstract

BBX transcription factors play vital roles in plant growth, development, and stress responses. Although BBX proteins have been studied in great detail in the model plant Arabidopsis, their roles in crop plants such as chrysanthemum are still largely uninvestigated. Here, we cloned CmBBX22 and further determined the function of CmBBX22 in response to drought treatment. Subcellular localization and transactivation assay analyses revealed that CmBBX22 was localized in the nucleus and possessed transactivation activity. Overexpression of CmBBX22 in chrysanthemum was found to reduce plant drought tolerance, whereas expression of the chimeric repressor CmBBX22-5RDX was found to promote a higher drought tolerance than that shown by wild-type plants, indicating that CmBBX22 negatively regulates drought tolerance in chrysanthemum. Transcriptome analysis and physiological measurements indicated the potential involvement of the CmBBX22-mediated ABA response, stomatal conductance, and antioxidant responses in the negative regulation of drought tolerance in chrysanthemum. Based on the findings of this study, we were thus able to establish the mechanisms whereby the transcriptional activator CmBBX22 negatively regulates drought tolerance in chrysanthemum via the regulation of the abscisic acid response, stomatal conductance, and antioxidant responses.

Introduction

Plants are adversely affected by a diverse range of biotic and abiotic stress factors, among which drought stress is potentially one of the most serious adverse factors disrupting plant metabolism, photosynthesis, and cell structure, thereby ultimately reducing plant growth [1, 2]. Consequently, it is of particular importance to study the response of plants to drought stress, thereby providing a theoretical basis for enhancing the drought tolerance of plants [3].

Zinc finger-containing proteins encode a family of plant-specific transcription factors that play significant roles in plant growth and development as well as response to environmental stimuli [4, 5]. The zinc finger family is one of the largest transcription factor families in plants [6, 7]. The BBX proteins are a subgroup of zinc finger transcription factors, carrying one or two conserved B-box domains in their N-terminus, and some members may also have a CCT (CONSTANS, CO-like, and TOC1) domain or a valine-proline (VP) motif in the C terminus [6, 8]. In Arabidopsis, it has been reported that the AtBBX family comprises 32 members, which can be divided into five subgroups based on their structural domains [8]. Among these, CONSTANS (CO/BBX1) has been identified as one of the most important factors involved in the regulation of flowering. Overexpression of CONSTANS in Arabidopsis has been demonstrated to result in earlier flowering than in wild-type (WT) plants under long-day conditions, whereas the findings of several studies have indicated that the BBX proteins play roles in the responses of plants to abiotic stress [9, 10]. In rice, the expression of OsBBX1, OsBBX2, OsBBX8, OsBBX19, and OsBBX24 has been observed to be strongly induced by abiotic stresses such as drought, cold, and salt stresses [11], and the overexpression of AtBBX21 in potato (Solanum tuberosum) plants has been found to promote a higher tolerance to water restriction, along with higher levels of chlorophyll and tuber yield than in WT plants [12]. Similarly, overexpression of the AtBBX24 (STO) gene in Arabidopsis results in a higher salt tolerance [13], whereas knockdown of CmBBX24 in chrysanthemum has the effect of reducing tolerance to freezing and drought stresses, which has been found to be associated with the downregulation of genes related to carbohydrate metabolism and soluble substances [14]. Furthermore, it has been reported that AtBBX18 is involved in thermotolerance via regulation of a set of heat-shock-responsive genes [15], whereas recent studies have revealed that CmBBX19 negatively regulates drought tolerance in chrysanthemum [16] and MdBBX7 endows drought tolerance in apple (Malus domestica) [17]. Collectively, the findings of these studies provide convincing evidence to indicate that BBX genes positively or negatively contribute to the regulation of abiotic stress responses in plant, particularly the response to drought.

Abscisic acid (ABA), one of the plant hormones, regulates multiple important biological processes, including plant development and stress response. Several studies have shown that BBX21
inhibits ABA-regulated seed germination through its binding to the promoter of ABI5 [18, 19]. In addition, CmBBX22 improves drought stress resistance in Arabidopsis by regulating the ABA signaling pathway [20]. A recent report showed that the BBX19-ABF3 complex plays an important role in modulating drought response in chrysanthemum in an ABA-dependent pathway [16]. Taken together, these studies suggest that some BBX proteins regulate plant development and stress response possibly through ABA signaling.

AtBBX22 contains two B-box domains near its N-terminus. Previous reports showed that AtBBX22 positively regulates photomorphogenesis in Arabidopsis through interacting with HYS and COP1, and its degradation was conducted by COP1-mediated ubiquitination [21, 22]. It has been reported that heterologous expression of CmBBX22 in Arabidopsis leads to delayed leaf senescence and improved drought tolerance [20]. However, the functions of CmBBX22 in chrysanthemum are still unknown.

Chrysanthemum (Chrysanthemum morifolium) is among the most widely cultivated ornamental species worldwide, the productivity and quality of which are materially influenced by environmental stress. In this study, we found that constitutive expression of CmBBX22 resulted in a heightened sensitivity to drought stress, whereas in contrast constitutively expressing the chimeric repressor CmBBX22-SRDX was found to promote a higher level of drought tolerance than that observed in WT plants. Moreover, transcriptome analysis and physiological measurements revealed that CmBBX22-mediated stomatal conductance, ABA response, and antioxidant responses are potentially involved in the negative regulation of drought stress in chrysanthemum. Collectively, our findings indicate that CmBBX22 acts in a negative role in the drought stress response in chrysanthemum.

Results
Subcellular localization and transcriptional activity of the CmBBX22 protein
CmBBX22 is a member of the B-box (BBX)-containing zinc finger transcription factor family [7, 20]. To investigate the subcellular localization of CmBBX22, we performed transient expression of CmBBX22 in Nicotiana benthamiana, by infiltrating plant leaves with a suspension of Agrobacterium tumefaciens carrying the 35S::GFP-CmBBX22 expression construct or the 35S::GFP control vector, together with 35S::D53-RFP vector as a nuclear localization marker. In plants transformed with the 35S::GFP plasmid, green fluorescence signals were detected in both cytoplasm and nucleus (Fig. 1A), whereas, in contrast, fluorescence was detected exclusively in the nuclei of leaf epidermal cells expressing the 35S::GFP-CmBBX22 plasmid (Fig. 1A), thereby indicating that, similar to other transcription factors, CmBBX22 is a nuclear-localized protein.

To examine the transcriptional activity of CmBBX22, we performed a reporter–effector transient expression assay. Initially, we generated a translational fusion construct, in which the protein-coding region of CmBBX22 was fused to the GAL4 DNA-binding domain (35S::GAL4DB-CmBBX22), whereas constructs containing the GAL4 DNA-binding domain linked to the strong activator AtARF5 (35S::GAL4DB-AtARF5) were used as a positive control [Supplementary Data Fig. S1] [23]. Intriguingly, we found that, compared with the GAL4-only negative control effector (35S::GAL4DB), both the 35S::GAL4DB-CmBBX22 and 35S::GAL4DB-AtARF5 effectors promoted an increase in luciferase (LUC) activity in transformed Arabidopsis mesophyll protoplasts (Fig. 1B and C), thereby indicating that CmBBX22 has transcriptional activator activity. Further confirmation that CmBBX22 functions as a transcription activator was obtained based on yeast two-hybrid (Y2H) Gold assays (Fig. 1D). Transformation of cells with a construct containing the C-terminal region of the CmBBX22 protein (residues 105–268), pGBK77-CmBBX22/C, was found to be sufficient to induce activation of the reporter, whereas, in contrast, no comparable expression was detected in cells harboring constructs containing the two Box domains [pGBK77-CmBBX22-Boxes (residues 1–104)], [pGBK77-CmBBX22/Box1 (residues 1–48 residues)] or [pGBK77-CmBBX22/Box2 (residues 51–104)] (Fig. 1D). These findings accordingly indicate that CmBBX22 is a transcription activator and that transcriptional activation is mediated via the C-terminal region of this protein.

The CmBBX22 gene negatively regulates drought tolerance in chrysanthemum
To further investigate the function of CmBBX22 in chrysanthemum, we generated overexpression lines of CmBBX22 in the cultivar ‘Jinba’ by introducing the plasmid pMDC43-CmBBX22, whereas chimeric repressor lines were generated by transforming with the 35S::CmBBX22-SRDX construct. Positive transgenic lines were identified by conducting a genomic PCR assay and quantitative real-time PCR (qRT–PCR) analysis [Supplementary Data Fig. S2A, Fig. 2A]. We accordingly found that the levels of CmBBX22 transcription were substantially higher in transgenic lines than in WT plants (Fig. 2A). On the basis of these findings, the CmBBX22-overexpressing lines CmBBX22ox-4 and CmBBX22ox-6 and the CmBBX22 chimeric repressor lines CmBBX22-SRDX-9 and CmBBX22-SRDX-13 were selected for subsequent analyses (Fig. 2A).

The heterologous expression of CmBBX22 in Arabidopsis has previously been demonstrated to enhance plant drought tolerance [20]. To examine the function of CmBBX22 in the regulation of drought tolerance in chrysanthemum, we subjected transgenic and WT chrysanthemums to a 16-day period of drought stress. We found that the CmBBX22ox lines were severely damaged by drought treatment, whereas CmBBX22-SRDX plants exhibited only slight damage compared with WT plants (Fig. 2B). Subsequent to drought treatment, plants were re-watered and continually cultured for a further 7 days, after which time the CmBBX22ox plants were found to have significantly lower survival compared with the WT control plants, whereas CmBBX22-SRDX plants showed comparatively higher survival (Fig. 2B and C). These observations thus tend to indicate that CmBBX22 negatively regulates drought stress tolerance in chrysanthemum.

As further confirmation of CmBBX22 function in the regulation of drought tolerance in chrysanthemum, we subjected transgenic and WT plants to a 20% FEG6000-simulated drought treatment. It was accordingly found that the degree of base-leaf wilting in CmBBX22ox plants was more pronounced than that observed in WT plants, whereas no significant impairment was observed in CmBBX22-SRDX plants (Fig. 2D), which is consistent with the previous observations that CmBBX22 negatively regulates drought tolerance in chrysanthemum.

Previous reports showed that the phytohormone ABA regulates drought stress responses and resistance in plants [20, 24]. To examine the effects of ABA on the CmBBX22-mediated drought response in chrysanthemum, we performed ABA treatment on detached chrysanthemum leaves. In WT plants, we found that ABA treatment maintained higher leaf water content for a longer period under water deficit conditions than the plants without ABA treatment, possibly due to reduced leaf transpiration by closing stomata [Supplementary Data Fig. S2B]. The largest
difference between ABA treatment and mock was observed at 6 h (Supplementary Data Fig. S2C). We then performed ABA treatment on the detached leaves from transgenic (CmBBX22ox-4 and CmBBX22-SRDX-9) and WT plants. We found that the CmBBX22ox plants had more severely wilted leaves compared with the WT control, whereas CmBBX22-SRDX plants wilted slightly (Supplementary Data Fig. S2D), indicating that the CmBBX22-mediated ABA response is potentially involved in the negative regulation of chrysanthemum drought tolerance.

**Global expression analysis of genes regulated by CmBBX22**

To gain more insight into the molecular mechanisms underlying the CmBBX22-mediated control of drought tolerance in chrysanthemum, we subsequently performed RNA-seq analysis using CmBBX22ox, CmBBX22-SRDX, and WT plants cultivated under normal growth conditions, for each type of which three biological replicates were collected for sequencing. Having initially removed adaptor sequences and low-quality reads from the raw sequence data, the remaining clean reads were aligned to the Chrysanthemum seticuspe genome release CSE_r1.0 sequence [25] using the kallisto program [26] (Supplementary Data Table S2). Verification of the similarity of three biological replicates of the WT and CmBBX22ox plants confirmed that the data were reproducible (Fig. 3A). However, one of the CmBBX22-SRDX replicates was found to have large variation compared with the other two replicates (Fig. 3A), and thus we performed subsequent analyses using only replicates 2 and 3. Analysis of the expression of Cs3_sc004370.1_g0600.1 (CmBBX22) obtained by RNA-seq revealed that, in line with the qRT–PCR results (Fig. 2A, Supplementary Data Fig. S3A and B), the transcriptional level of CmBBX22 in transgenic plants was considerably higher than that in WT plants.

Having characterized the expression of CmBBX22 in response to drought, we proceeded to identify differentially expressed genes (DEGs) using DEseq2 methods [27]. In total, we identified 5942 and 2085 DEGs by comparing between CmBBX22ox and WT and between CmBBX22-SRDX and WT plants, respectively (Fig. 3B and C; Supplementary Data Table S3). To validate the expression of genes in transgenic and WT plants, a couple of genes were selected for qRT–PCR, including the drought-responsive genes Cse_sc006945.1_g030.1 (AT1G19640, JASMONIC ACID CARBOXYL METHYLTRANSFERASE) and Cse_sc000276.1_g070.1 (AT3G47780, ATP-BINDING CASSETTE A7) [28, 29]. qPCR and Integrative Genomics Viewer data showed that the gene expression patterns were consistent with the expression levels obtained based on RNA-seq analysis (Fig. 3D and E; Supplementary Data Fig. S3C and D).

To gain a functional insight into these DEGs, we performed Gene Ontology (GO) analysis, the results of which revealed an enrichment of GO terms in multiple biological processes and molecular functions, including water transmembrane activity, water channel activity, and response to abiotic stimulus, among genes differentially expressed between CmBBX22ox and WT (Fig. 3F), and responses to water deprivation, stress, and desiccation among those differentially expressed between CmBBX22-SRDX and WT (Fig. 3G). In addition, we also found that the chlorophyll binding, response to hormone, response to light, and cell death-associated pathways were enriched among the DEGs identified in both comparisons (Fig. 3F and G). Taken together,
these results indicate that CmBBX22 could influence pathways associated with stress and hormonal responses.

Given that the chlorophyll binding pathway was found to be enriched with DEGs identified in both CmBBX22ox versus WT and CmBBX22-SRDX versus WT comparisons (Fig. 3F and G), and the chlorophyll fluorescence parameter $F_v/F_m$ gave insight into photosynthetic capacity [30] and is one of the most commonly used indexes for resistance assessment [31], we further examined $F_v/F_m$ in transgenic and WT plants. We were unable to detect any significant differences in $F_v/F_m$ ratios among CmBBX22ox, CmBBX22-SRDX, and WT lines (Fig. 4; Supplementary Data Fig. S4), indicating that CmBBX22 has no direct regulatory activity with respect to PSII activity in photosynthesis. In response to PEG6000 treatment, however, we did observe higher and lower amounts of $F_v/F_m$ in CmBBX22-SRDX and CmBBX22ox plants, respectively, compared with those in WT control plants (Fig. 4; Supplementary Data Fig. S4), consistent with the negative functions of CmBBX22 in response to drought treatment.

**CmBBX22-mediated stomatal conductance is potentially involved in the negative regulation of chrysanthemum drought tolerance**

To further examine the function of CmBBX22-regulated genes, we performed GO analysis of those genes identified as being upregulated in CmBBX22ox plants and downregulated in CmBBX22-SRDX plants. In line with the findings obtained for GO enrichment of all DEGs (Fig. 3F and G), we observed the enrichment of GO terms associated with water, stress, light, and hormone responses and cell death (Fig. 5A and B). Previous studies have shown that potato plants in which the Arabidopsis AtBBX21 gene is heterologously expressed are characterized by higher stomatal conductance associated with an increase in the size of the stomatal apertures [32]. Consistent with this observation, we similarly found that the stomatal movement pathway was enriched with genes that were upregulated in CmBBX22ox (Fig. 5A), thereby indicating that CmBBX22 negatively regulates drought tolerance, potentially by modulating stomatal movement.
Figure 3. Global expression analysis of genes regulated by CmBBX22. (A) Heat map of correlation values for pairwise comparisons between samples. The correlation analysis was performed by DEseq2. OX4 indicates the CmBBX22ox-4 transgenic plant; SR9 indicates the CmBBX22-SRDX-9 plant. (B, C) Volcano plots of gene expression changes in CmBBX22ox versus WT (B) and CmBBX22-SRDX versus WT (C). OX4 indicates the CmBBX22ox-4 transgenic plant; SR9 indicates the CmBBX22-SRDX-9 plant. DEGs were selected by q < 0.05 and |log2 (fold change)| > 1. The x-axis shows the fold change in gene expression between transgenic and WT plant samples, and the y-axis shows the statistical significance of the differences. Colors represent different genes: gray for genes without significantly different expression, blue for significantly downregulated genes, and red for significantly upregulated genes. (D) Genome browser traces of RNA-seq results of DEGs in CmBBX22ox, CmBBX22-SRDX, and WT plants. Chrysanthemum EF1α was used as the reference gene for normalization. Error bars indicate the standard deviation (n = 3). Different letters indicate statistical differences determined by Duncan’s test (P < 0.05). (F, G) GO analysis using all the DEGs between CmBBX22ox and WT (F) and CmBBX22-SRDX and WT (G). RNA-seq analyses were performed using CmBBX22ox, CmBBX22-SRDX, and WT plants cultivated under normal growth conditions. GO analyses were conducted using 5942 and 2085 DEGs that were identified by comparisons between CmBBX22ox and WT and between CmBBX22-SRDX and WT plants, respectively. The bubble plot shows enrichment for GO pathways.
To further examine the putative influence of CmBBX22 on stomatal movement, we examined stomatal conductance in CmBBX22 transgenic and WT plants, and accordingly detected higher and lower stomatal conductance in CmBBX22ox and CmBBX22-SRDX, respectively, than in WT plants (Fig. 5C and D), thus indicating that CmBBX22 plays a role in promoting stomatal opening. The findings of recent studies have also revealed that plants can optimize their CO₂ uptake for photosynthesis and minimize water loss by altering the size of stomatal pore apertures [33], and thus we further investigated the rates of water loss in transgenic and WT plants. Observations revealed that CmBBX22ox and CmBBX22-SRDX plants show higher and lower rates of water loss, respectively, compared with those recorded in WT plants (Fig. 5E). In this regard, relative water content (RWC) is considered to be one of the most significant indices of dehydration tolerance [34], and hence we analyzed RWC in plants subjected to drought treatment. Whereas in untreated plants we found no significant differences between transgenic and WT plants with respect to RWC, after 72 h of PEG6000 treatment the CmBBX22-SRDX and CmBBX22ox transgenic plants were found to have higher and lower RWCs, respectively, than WT plants (Supplementary Data Fig. 55).

Collectively, these observations thus indicate that CmBBX22-induced stomatal opening leads to high rates of water loss, thereby contributing to a reduction in the drought tolerance of chrysanthemum.

The CmBBX22-mediated antioxidant response is potentially involved in the negative regulation of chrysanthemum drought tolerance

Exposure of plants to drought stress leads to excess production of toxic highly reactive oxygen species (ROS) that cause damage to plant cells. To scavenge ROS, plants deploy an efficient antioxidant defense system comprising non-enzymatic antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) [35–37]. In the present study, we found that the response to oxidative stress pathway was enriched with genes identified as being upregulated in CmBBX22ox plants (Fig. 5A), thereby indicating that CmBBX22 may play a role in regulating the response to oxidative stress.

To assess the veracity of this supposition, we proceeded to examine SOD and POD enzyme activities, which revealed that whereas SOD activity was not affected by CmBBX22 under normal conditions, POD activity was elevated in CmBBX22-SRDX plants (Fig. 6A and B). The activities of both SOD and POD were found to be altered in response to drought treatment, with higher and lower levels in CmBBX22-SRDX and CmBBX22ox plants, respectively, compared with the activities in WT plants (Fig. 6A and B). These observations would thus tend to indicate that CmBBX22 negatively regulates drought tolerance by reducing ROS scavenging ability.

In plants, the accumulation of proline is a well-established metabolic response to drought and other stresses [38], which contributes to providing protection against different ROS [39]. On examining proline contents in transgenic and WT plants subjected to drought, we detected higher and lower levels of proline accumulated in CmBBX22-SRDX and CmBBX22ox plants, respectively, compared with those in WT plants, whereas no significant differences were detected under normal conditions (Fig. 6C). These findings indicate that CmBBX22 may also contribute to the negative regulation of drought tolerance in chrysanthemum by modulating accumulation of the antioxidant proline.

**Discussion**

Zinc finger-homeodomain genes comprise a relatively large family of transcription factors in plants (~15% of the total), with 32, 29, and 64 members having been identified in *Arabidopsis*, rice, and apple, respectively [22, 40, 41], and play a central role in plant growth and development and the response to environmental stimuli [8]. The BBX family is a subgroup of the zinc finger-homeodomain proteins, characterized by the presence of one or two N-terminal B-box domains [8]. Among these BBX proteins, it has been reported that members of the fourth subfamily play an important role in the responses to abiotic stress [13–15]. AtBBX22, which contains two N-terminal B-box domains, has previously been reported to positively regulate photomorphogenesis in *Arabidopsis* via its interaction with HY5 and COP1, and is degraded by COP1-mediated ubiquitination [21, 22]. Furthermore, heterologous expression of chrysanthemum CmBBX22 in *Arabidopsis* has been found to promote delayed leaf senescence and enhance drought tolerance [20], while in this study we found CmBBX22 to be a negative regulator of drought stress tolerance, mediated via regulation of stomatal conductance and antioxidant responses in chrysanthemum.

CmBBX22 is a transcription activator and transcriptional activation was mediated via the C-terminal region of this protein. In previous reports, the fragment between the B-box and CCT domains in BBX4, BBX8, and BBX16/COL7 had transcriptional activation activity in yeast cells [42–44], suggesting that an unidentified motif might reside within the fragment regions or C-terminal region. Recent research has found that the functional diversity within B-box proteins in structural group IV was caused by divergence of the C-terminal domains in *Arabidopsis* [45]. The motifs and residues in the C-terminal regions of BBX proteins need further research. Previous reports showed that AtARF5 is an activator with super-strong activities and is usually used as a positive control for the activity analysis of transcriptional activators [46, 47]. 35S::GAL4DB-AtARF5 protoplasts showed high LUC activity, indicating the success of our LUC assay. We found that the LUC
activity in Arabidopsis mesophyll protoplasts transformed with 35S::GAL4DB-CmBBX22 was higher than in the negative control, indicating that CmBBX22 is a transcription activator. We also observed a lower LUC activity in 35S::GAL4DB-CmBBX22 protoplasts than in the 35S::GAL4DB-AtARF5 positive control, suggesting that the transcription activity of CmBBX22 is lower than AtARF5, which is consistent with the reports that different transcriptional activators trigger gene expression at different levels [23, 48].

We established that overexpression of CmBBX22 in chrysanthemum leads to a reduced tolerance to drought. Notably, this differs from the drought response phenotype obtained by heterologous expression of CmBBX22 in Arabidopsis, the transformed plants of which showed enhanced drought tolerance [20]. Although seemingly counterintuitive, these observations are consistent with previously reported findings indicating that heterologous transformation of Arabidopsis with chrysanthemum genes sometimes leads to an opposite phenotype. For example, overexpression of CmMYB2 in chrysanthemum results in earlier flowering, whereas heterologous expression of CmMYB2 in Arabidopsis delays flowering [46, 49]. These observations would therefore tend to imply that chrysanthemum and Arabidopsis differ with respect to mechanisms underlying the regulation of plant development and stress responses. Unlike chrysanthemum, which is a perennial plant, Arabidopsis is an annual with a rapid life cycle and produces...
seeds within ∼8 weeks. Consequently, it might be anticipated that chrysanthemums suffer higher levels of abiotic and biotic stresses during their life cycle. Thus, it can be speculated that, to adapt to adverse conditions, plants have evolved species-specific mechanisms that optimize plant growth to provide varying degrees of stress tolerance. However, further research is needed in this regard to elucidate the underlying mechanisms.

ROS play vital roles in the response to biotic and abiotic stimuli in plants. On encountering different stressful conditions, plants rapidly accumulate common ROS as a first layer of defense [50, 51]. However, when produced in excessive amounts, ROS can also cause irreversible cell damage. To counter these adverse effects, plants have evolved ROS scavenging mechanisms comprising two pathways involving non-enzymatic antioxidants and enzymatic components. Enzymatic components include SOD, CAT, APX, NADH, glutathione reductase (GR), guaiacol peroxidase (GPX), and monodehydroascorbate reductase, whereas glutathione (GSH), ascorbic acid, carotenoids, and osmolyte-proline are among the non-enzymatic antioxidants [35]. Constitutive expression of OsGSTU4 (glutathione S-transferase) in Arabidopsis has been demonstrated to enhance the tolerance of transgenic lines to salinity and oxidative stresses by reducing the accumulation of ROS [52], whereas overexpression of a POD gene (AtPER64) in tobacco has been shown to reduce the root accumulation of aluminum and ROS [39]. In the present study, we found that the activities of both POD and SOD were significantly enhanced in CmBBX22-SRDX transgenic plants compared with WT plants (Fig. 6A and B), indicating that CmBBX22 plays a role in the negative regulation of ROS scavenging, and further contributes to the negative regulation of drought tolerance. Proline is an osmoregulation substance, which will be accumulated largely under drought stress so as to reduce the damage to cells caused by drought and improve the drought tolerance of plants. We found that accumulation of proline in CmBBX22-SRDX plants was significantly higher than that detected in WT plants (Fig. 6C). These findings thus indicate that CmBBX22 negatively regulates the antioxidant defense system and further diminishes the sensitivity of chrysanthemum to drought stress.

ROS regulate stomatal movements [53]. Besides ROS, it has been widely reported that many other factors could contribute to stomatal movement, including ABA, ethylene, nitric oxide (NO), and hydrogen sulfide (H2S) [54–56]. In addition, a recent report showed that high ROS levels could lead to ABA insensitivity, forming a feedback repression of continuously activated ABA signaling in guard cells [56]. ABA signaling plays a central role in the regulation of stomatal movements, particularly under water-deficit conditions. For example, HAS1 is a negative regulator of ABA signaling and the has1 mutant showed an ABA-hypersensitive stomatal closure phenotype [57]. The expression of HAS1 was induced by CmBBX22 in our transcriptome analysis, indicating possible roles of ABA signaling in CmBBX22-mediated stomatal opening.

In conclusion, CmBBX22-mediated stomatal conductance may be controlled by integration effects of ROS, ABA signaling, and other factors. In the future, experiments related to functional verification of these factors in stomatal conductance are needed to further identify the underlying molecular mechanism of CmBBX22-mediated stomatal conductance.

In summary, we identified a BBX family gene, CmBBX22, in chrysanthemum and established that CmBBX22 regulates drought stress responses in chrysanthemum. Transcriptome analysis and physiological measurements revealed that the CmBBX22-mediated ABA response, stomatal conductance, and antioxidant responses potentially contribute to the negative regulation of drought tolerance. Collectively, the findings of this study
enable us to propose mechanisms whereby the transcriptional activator CmBBX22 negatively regulates drought tolerance in chrysanthemum.

Materials and methods

Plant growth conditions

The cut flower chrysanthemum cultivar 'Jinba' was obtained from the Chrysanthemum Germplasm Resource Conservation Center in Nanjing Agricultural University (Nanjing, China). Cuttings were planted in plug trays, and later the seedlings were transplanted in a 1:1 (v/v) mixture of vermiculite and peat in a greenhouse (day/night temperature 25/18°C, photoperiod 16 h, light intensity 120 μmol m⁻² s⁻¹, relative humidity 70%). We selected transgenic lines and WT plants that were at the same developmental stages for drought treatments. At first the plants were given adequate water and then water was withheld for 16 days. We next calculated the survival rate of the transgenic lines and the WT plants after 7 days' recovery. For PEG6000-simulated drought treatment, transgenic and WT cuttings at the 8- to 10-leaf stage were treated with 20% w/v PEG6000, while those of control cuttings were treated with water [58, 59]. After treatment, plants were maintained in a greenhouse. Two leaves at the bottom of three seedlings were collected for further analysis.

Subcellular localization analysis of CmBBX22

The CmBBX22 ORF sequence was amplified using the primers covering CmBBX22 open reading frame (ORF) regions and the construction of p35S::GFP-CmBBX22 was performed as described previously [20]. For transient expression assays, Agrobacterium strain GV3101 carrying the constructs was infiltrated into 5- to 6-week-old N. benthamiana tobacco leaves. The tobacco plant was then cultured in darkness for 1 day followed by light treatment for 2 days. The green fluorescent protein (GFP) fluorescence signal was detected by laser confocal microscope (Zeiss, LSM800).

Transcriptional activity analysis of CmBBX22

The CmBBX22 ORF and amplicons were obtained using the Phusion High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA, USA) using primer pairs containing EcoRI and BamHI sites. A set of pGBK7-CmBBX22 fusions were generated by T4 DNA ligase (Takara), including the pGBK7-CmBBX22 construct (with full-length ORF sequence), pGBK7-CmBBX22/Box1 construct, pGBK7-CmBBX22/Box2 construct, pGBK7-CmBBX22/Boxes (including CmBBX22/Box1 and CmBBX22/Box2), pGBK7-CmBBX22/C construct, pCL1 (positive control), and pGBK7 (negative control). The plasmids described above were transformed into Y2H Gold strain (Clontech) [60]. Transformants carrying either pGBK7-CmBBX22, CmBBX22 residues or pGBK7 were cultured on SD−Leu medium at 30°C, whereas the positive control pCL1 was cultured on SD−His medium with or without X-α-gal at 30°C. All primers used for this study are listed in Supplementary Data Table S1.

For the LUC assay, we recombined the pENTR™-A-CmBBX22 plasmid into the p35S::GAL4DB vector by LR recombination. The preparation and transformation of Arabidopsis protoplasts were performed as previously described [61]. Generally, 10 μg of p35S::GAL4DB-AARFP, p35S::GAL4DB, or p35S::GAL4DB-CmBBX22 plasmid was co-transformed with 5 × GAL4-LUC reporter gene into Arabidopsis protoplasts. After overnight incubation, the fluorescence values were obtained according to methods described previously [62].

Transformation of chrysanthemum

The overexpression vector pMDC43-CmBBX22 and entry vector pENTR™-A-CmBBX22 were as described previously [20], and the CmBBX22 ORF from the last one was recombined into the pDEST_35S_SRDX_BCKH vector [63] via LR Clonase™ II enzyme mix (Invitrogen) to get the 35S::CmBBX22-SRDX construct as a chimeric repressor. For plant transformation, we introduced the 35S::CmBBX22 and 35S::CmBBX22-SRDX plasmids into Agrobacterium strain EHA105, and then generated transgenic chrysanthemum as described by Li et al. [64].

Measurement of stomatal aperture

The leaves of WT and transgenic plants were harvested, and the epidermis close to the mid-vein was taken from the abaxial side. The epidermis was suspended on MES-KCl solution (10 mM MES, 50 mM KCl, pH 6.2) under lights for 2 hours at 22°C. Stomatal apertures of 24 guard cells in the epidermis of the abaxial surface were examined under a microscope, and three biological replicates were analyzed. The width and length of the stomatal aperture of guard cells per sample were measured using Olympus LAS V4.11 software. All measurements were performed between 8 a.m. and 12 noon, at a temperature of 22°C [65].

Relative water content and water loss rate measurements

The transgenic chrysanthemum and WT seedlings at the 8- to 10-leaf stage were selected, and their roots were treated with 20% w/v PEG6000 solution. The third leaf (from the top) of three seedlings was collected at 0 h and 72 h of PEG treatment. The fresh weight of these leaves was measured. The leaves were then placed in deionized water for 24 hours at 22°C and re-weighed to give the turgid weight. Next, these leaves were baked at 65°C for 48 hours, then the dry weight was measured. RWC was calculated according to the method described previously [66]. Three biological replicates were performed.

The third fully expanded leaves from the top of CmBBX22ox, CmBBX22-SRDX, and WT plants were collected to estimate leaf water loss using the detached leaf method. Leaf fresh weight was measured immediately after detaching from the seedlings, then the leaves were stored at room temperature and weighed at 0, 2, 4, 6, 8, 10, and 12 hours after detaching. Experiments were performed with three biological replicates. The percentage of water loss was calculated as described by Ren et al. [67].

Measurement of chlorophyll fluorescence parameters

All the expanded leaves at the bottom were collected. The chlorophyll fluorescence parameters were measured by pulse-amplitude modulation fluorometer (IMAGING-PAM, Walz, Germany) at room temperature (25°C), according to the method described by Su et al. [31]. The value of maximal photochemical efficiency (Fv/Fm) was calculated [31].

Measurement of proline and antioxidant enzyme activity

SOD and POD activities and proline content were measured according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

RNA extraction and RNA-seq analysis

The fifth unfolded leaves of CmBBX22-overexpressing line CmBBX22ox-4, the CmBBX22 chimeric repressor line CmBBX22-
SRDX-9, and WT plants were collected at 30 days after transplanting. Total RNA was extracted with a Quick RNA Isolation Kit (Waryong, Beijing, China) and subjected to the Illumina sequencing platform at the Beijing Genomics Institute (Shenzhen, China) using an Illumina HiSeq™ 2000 instrument. Experiments were performed with three biological replicates. Adaptor sequences and low-quality reads were removed, and the clean data were then aligned to the C. seticuspe genome release CSE_r1.0 [25] using the kallisto [26] program. DEGs were identified using DEseq2 [27]. Genes showing $q < 0.05$ and $|\log 2(\text{fold change})| > 1$ were considered to be differentially transcribed. Homologous genes of chrysanthemum in Arabidopsis were downloaded from the Mum Genome And Research Database Entry (http://mum-garden.kazusa.or.jp/), and then GO analysis for these genes was performed using the online database agriGO [68]. Heat maps and Venn diagrams were generated using R (version 4.0.4).

Quantitative real-time PCR validation

The primers used for validation were designed using Primer 5.0 software, and qRT–PCR was performed as previously described [69, 70]. The EF1α gene (GenBank AB548817.1) was selected as a reference [71]. Relative abundance was calculated using the 2−ΔΔCt method [72]. The experiments were performed with three biological replicates, and each sample was examined with three technical replicates. All primers used in this study are listed in Supplementary Data Table S1.

Abscisic acid treatment of detached chrysanthemum leaves

The third fully expanded leaves from the top of WT plants and the transgenic plants were detached and put into 2-mL EP tubes containing 2-mL water in the presence or absence of 50 μM ABA (Sigma–Aldrich, St Louis, MO, USA) for pretreatment for 12 hours in darkness, then transferred to empty 2-mL EP tubes as described previously [73]. Leaves were photographed at 0 and 6 hours respectively, and the water loss rates of the mock and ABA treatment groups were measured as described above. At least three replicated experiments were performed independently.

Statistical analysis

Student’s t-test was used to determine significant differences in the results from Arabidopsis mesophyll protoplasts and water loss assays. Duncan’s multiple-range test was used for the analysis of significant differences in other results. All statistical analyses were performed with SPSS v19.0 (SPSS Inc., Chicago, IL, USA).

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Author contributions

J.J. and L.W. conceived and designed the experiments; H.C. and Y. (Ye) L. performed most of the experiments; J.L. A.S. and Y. (Yanan) L. provided technical support; C.W., S.C. and F.C. provided conceptual advice; P.C. contributed to plant transformation; H.C., Y.L., J.J., and L.W. analyzed the data and wrote the manuscript.

Availability of data and materials

The data supporting this work are available in the paper and its supplementary information files. The data generated and analyzed in the study are available from the corresponding author upon request.

Conflict of interest

The authors declare that they have no conflicts of interest.

Supplementary data

Supplementary data is available at Horticulture Research online.

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