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# Identification and functional characterization of the *BBX24* promoter and gene from chrysanthemum in Arabidopsis

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**Abstract** The B-box (BBX) family is a subgroup of zinc finger transcription factors that regulate flowering time, light-regulated morphogenesis, and abiotic stress in Arabidopsis. Overexpression of *CmBBX24*, a zinc finger transcription factor gene in chrysanthemum, results in abiotic stress tolerance. We have investigated and characterized the promoter of *CmBBX24*, isolating a 2.7-kb *CmBBX24* promoter sequence and annotating a number of abiotic stress-related *cis*-regulatory elements, such as DRE, MYB, MYC, as well as *cis*-elements which respond to plant hormones, such as GARE, ABRE, and CARE. We also observed a number of *cis*-elements related to light, such as TBOX and GBOX, and some tissue-specific *cis*-elements, such as those for guard cells (TAAAG). Expression of the *CmBBX24* promoter produced a clear response in leaves and a lower response in roots, based on β-glucuronidase histochemical staining and fluorometric analysis. The *CmBBX24* promoter was induced by abiotic stresses (mannitol, cold temperature), hormones (gibberellic acid, abscisic acid), and different light treatments (white, blue, red); activation was measured by fluorometric analysis in the leaves and roots. The deletion of fragments from the 5′-end of the promoter led to different responses under various stress conditions. Some Cm*BBX24* promoter segments were found to be more important than others for regulating all stresses, while other segments were relatively more specific to stress type. D0-, D1-, D2-, D3-, and D4-pro*CmBBX24*::*CmBBX24* transgenic Arabidopsis lines developed for further study were found to be more tolerant to the low temperature and drought stresses than the controls. We therefore speculate that *CmBBX24* is of prime importance in the regulation of abiotic stress in Arabidopsis and that the *CmBBX24* promoter is inductive in abiotic stress conditions. Consequently, we suggest that *CmBBX24* is a potential candidate for the use in breeding programs of important ornamental plants.

**Keywords** Chrysanthemum · *BBX24* · Stress tolerance · Promoter activity

## Introduction

The B-box protein family is a class of zinc finger transcription factors that play various roles in various species (Yamawaki et al. 2011; Huang et al. 2012; Crocco and Botto 2013; Wang et al. 2013). They are characterized by the presence of one or two B-box motifs in the N-terminal domain, either alone or in combination with a CCT domain (Khanna et al. 2009; Huang et al. 2012). The B-box itself is a zinc-binding domain consisting of conserved Cys and His residues (Klug and Schwabe 1995; Torok and Etkin 2001) which form multiple finger-like protrusions that can bind metals such as zinc. Proteins with Zn finger domains often contain other specialized interaction domains and have been recognized to bind DNA, RNA, or proteins, as is thought to also be the case for other Zn finger proteins, such as the B-box proteins (Borden et al. 1995; Rushton et al.1995). B-box-containing proteins with one or two

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B-boxes in the N-terminal end are all transcription factors and termed BBX according to a uniform nomenclature in Arabidopsis (Kumagai et al. 2008a, b; Khanna et al. 2009).

In Arabidopsis, the BBX family has 32 members (Kumagai et al. 2008a, b; Khanna et al. 2009) which are divided into five structure groups based on the number and the sequence features of their B-box domains and whether they contain the CCT domain. Structure group I contains six members (BBX1–6), each of which contains two B-box domains and a CCT domain. Group II has seven members (BBX7–13) which are similar to group I transcription factors, with differences only in their second B-box domains (Chang et al. 2008). Group III has four members (BBX14–17), and they contain only one B-box domain and a CCT domain. The best-known BBX1 protein is CONSTANS (CO), and BBX2–17 are CO-LIKE (COL) proteins. Group IV has eight members (BBX18–25), each of which contains two B-box domains at the C-terminal end but no CCT domain at the N-terminal end. Finally, Group V has seven members (BBX26–32), all of which contain just a single B-box domain (Khanna et al. 2009).

Flowering induction by long days (LD), i.e., exposure to light for longer than the specific plant's critical day length, involves an endogenous clock (circadian clock) that interacts with light cues. Light—more specifically photoperiod—is involved in the regulation of biosynthesis of the gibberellic acids (GAs), and photoperiod effects on flowering are at least partially separable (Blázquez et al. 2002).

The expression profiles of many members of the BBX protein family have been reported to be under the control of circadian rhythm. In terms of function, some members in structure groups I–III play roles in regulating flowering time (Park et al. 2011). *CO* (BBX1) plays an important role in the photoperiodic regulation of flowering, promoting flowering under LD conditions, but having no effect on flowering regulation under short day (SD) conditions (Huang et al. 2012; Putterill et al. 1995). It also promotes light-regulated stomatal opening (Kinoshita et al. 2011; Ando et al. 2013). Changes in the expression of *BBX2* (*COL1*) and *BBX3* (*COL2*) have little effect on flowering time, but the overexpression of *BBX2* shortens the duration of two distinct circadian rhythms (Ledger et al. 2001). Under both SD and LD conditions *COL3* (*BBX4*) positively regulates photomorphogenesis and enhances root growth and negatively regulates flowering (Datta et al. 2006). *BBX6* (*COL5*) specifically induces flowering under SD conditions by promoting *FLOWERING LOCUS T* (*FT* expression (Hassidim et al. 2009), while *BBX7* (*COL9*) works as a repressor of flowering under LD conditions by reducing *CO* and *FT* expression.

The members of group IV mainly participate in early photomorphogenesis and shade-avoidance syndrome (SAS) (Park et al. 2011, Bowler et al. 2013, Gangappa and Botto 2014). *BBX18* (*DBB1a*), *BBX19* (*DBB1b*), *BBX24* (*STO*) and *BBX25* (*STH1*) have been observed to be negative regulators of light signaling (Holm et al. 2001; Indorf et al. 2007; Chang et al. 2008; Kumagai et al. 2008a, b; Khanna et al. 2009; Wang et al. 2011), while *BBX21* (*STH2*) and *BBX22* (*LZF1*/*STH3*) are positive regulators of light signaling (Datta et al. 2007, 2008; Chang et al. 2011). *BBX32*, a member of BBX protein group V, which contains just one B-box motif, modulates light signaling and has also been reported to be involved in affecting flowering time and early photomorphogenesis (Holtan et al. 2011; Park et al. 2011).

The expression of *BBX24* is controlled by circadian rhythm and is affected by phytohormones and environmental factors. Phenotypic differences have been recorded in *BBX24* mutants from seed germination to flowering (Li et al. 2014). During the de-etiolation process, BBX24 accumulates in the first hour of exposure to light, but this accumulation is temporary, and the level of BBX24 decreases after prolonged exposure to light. Physical interaction with other proteins is also required for BBX24 degradation under light conditions in order for normal photomorphogenesis to occur (Yan et al. 2011). *BBX24* and *BBX25* have interact in an epistatic manner through which they enhance each other's functions; however, they can also work independently in the regulation of seedling photomorphogenesis (Gangappa et al. 2013). Both *BBX24* and *BBX25* suppress the function of *LONG HYPOCOTYL 5* (*HY5*), a positive regulator of photomorphogenesis, by heterodimer formation with *HY5* and thereby reduce the transcriptional activity of *HY5* on target genes, such as *CHI* and *CHS* (Gangappa et al. 2013; Gangappa and Botto 2014), which suggests that *BBX24* and *BBX25* work as *HY5* transcription co-repressors, likely as co-repressors of *HOMOLOG OF HY5* (*HYH*) (Gangappa et al. 2013).

*BBX24* mutant plants develop significantly a shorter hypocotyl under shade conditions, while *BBX25* mutant plants in a partially redundant manner further enhance *BBX24* phenotypes (Gangappa et al. 2013). As the *BBX24*, *BBX25*, and *COP1* triple mutant plants resemble the *Constitutive Photomorphogenesis1* (*COP1*) phenotypes, it has been speculated that the short hypocotyl phenotypes of the double *BBX24*, *BBX25* mutants under shade conditions are completely *COP1*-dependent (Gangappa et al. 2013), which suggests that under shade conditions the BBX proteins are involved in the *COP1* signaling pathway (Gangappa and Botto 2014).

The *BBX24* also negatively regulates UV-B signaling, attenuates the accumulation of *HY5* and suppresses transcriptional activity, most likely by constructing an inactive heterodimer with *HY5* (Jiang et al. 2012). Interestingly, BBX24 protein physically interacts with *RCD1* (*RADICAL-INDUCED CELL DEATH1*), another UV-B signaling

regulator that inhibits *BBX24* expression (Jaspers et al. 2009; Jiang et al. 2009), suggesting that *BBX24* is involved in the fine-tuning UV-B photomorphogenic responses through a negative feedback mechanism (Oravecz et al. 2006; Gangappa and Botto 2014). *BBX24* has been recently observed to plays a positive role in the control of shade elongation responses (Crocco et al. 2015) and to affect the expression of key flowering time genes *FLC* and *SOC1/FT* separately and thus regulate flowering (Li et al. 2014).

The BBX proteins in addition to their functions in plant growth and development are also involved in the induction of biotic and abiotic signaling pathways. For example, Arabidopsis lines with sub-expression of BBX19 show increased thermo-tolerance, whereas Arabidopsis lines overexpressing this protein show reduced thermo-tolerance (Wang et al. 2013). *BBX24* has been observed to be involved in signaling in response to salt stress (Lippuner et al. 1996; Nagaoka and Takano 2003), and the BBX24 protein was actually first isolated as a SALT TOLERANT gene (*STO*) in a screening process aimed at identifying clones of Arabidopsis cDNA that confer enhanced salt tolerance in *Saccharomyces cerevisiae* (yeast) salt-sensitive calcineurin mutants. The cDNA of *BBX24* (*STO*) complements the phenotype of the yeast calcineurin-deficient mutant and increases the salt tolerance capacity of the wild-type (WT) phenotype (Lippuner et al. 1996). *BBX24* overexpression in transgenic Arabidopsis plants also enhances salt tolerance as compared to WT Arabidopsis plants (Nagaoka and Takano 2003). A significant increase in root growth was observed in *BBX24* transgenic Arabidopsis plants grown on a medium supplemented with NaCl concentrations (50 and 100 mM), but the expression of *BBX24* was not altered by the salt, suggesting that *BBX24* probably indirectly caused the effects. Interesting, *BBX24* directly interacts with the H-protein promoter binding factor1 (*HPPBF-1*), which is a transcription factor regulating response to salt (Nagaoka and Takano 2003).

To date only limited data are available on the role of BBX proteins in hormone signaling pathways. *BBX18* has been observed to be involved in the GA signaling pathway (Wang et al. 2011). Phenotypic and molecular investigations have revealed that *BBX18* promotes hypocotyl growth by increasing the levels of bioactive GA. More specifically, *BBX18* enhances expression of the *GA3ox1* and *GA20ox1* metabolic genes and suppresses expression of the *GA2ox1* and *GA2ox8* catabolic genes under light conditions (Wang et al. 2011; Gangappa and Botto 2014). A microarray database study of rice (*oryza sativa*) plants revealed that the expression of 11 *OsBBX* genes changed with the addition of phytohormones [auxin (1-naphthaleneacetic acid), GA (GA3), and cytokinin (kinetin)] (Huang et al. 2012). Most of these transcripts harbor hormone-responsive *cis*-acting elements in their promoters. These authors proposed that *OsBBX* genes are likely involved in hormone signaling as transcriptional regulators. However, further investigations are required to clearly demonstrate the roll of BBX proteins in hormone-signaling pathways (Gangappa and Botto 2014).

BBX proteins play important roles in other plants besides Arabidopsis. For example, in rice (*Oryza sativa*), *OsBBX5* (*OsCOL4*) negatively regulates flowering in under both SD and LD conditions (Lee et al. 2010), but *OsBBX18* (*Hd1*) positively regulates flowering under SD conditions and negatively under LD ones (Yano et al. 2000), and *OsBBX27* (*OsCO3*) controls flowering by negatively regulating *FT*-like genes under SD conditions (Kim et al. 2008). In barley (*Hordeum vulgare*), *HvCO1*, a circadian clock gene, positively regulates flowering under both SD and LD conditions (Campoli et al. 2012). The overexpression of *BBX1* in potato (*Solanum tuberosum*) suppresses tuber formation (Martínez-García et al. 2002), the overexpression of the beetroot gene *BvCOL1* in Arabidopsis enhances flowering under LD conditions (Chia et al. 2008), and overexpression of the grape (*Vitis vinifera* L.) gene *VvCO* promotes flowering in response to light (Almada et al. 2009), while *VvZFPL1* overexpression in Arabidopsis enhances abiotic stress tolerance but negatively regulates photomorphogenesis (Takuhara et al. 2010). Overexpression of the Arabidopsis *BBX32* gene in soybean (*Glycine max* L.) enhances grain yield (Preuss et al. 2012).

Chrysanthemum is an important ornamental plant, but its sensitivity to cold and dehydration stresses has been a key factor limiting its cultivation. It is a typical SD plant, and its annual production mainly depends on the artificial regulation of photoperiod. The stress-inducible promoter in other ornamental plants has never been reported in chrysanthemum despite extensive research in Arabidopsis. The chrysanthemum *CmBBX24 gene* contains two B-box domains in the N terminal end without a CCT domain in the C-terminal end and belongs to the structure subgroup IV of the BBX family. In a previous study, we determined that *BBX24* protein from chrysanthemum coordinates plant responses to abiotic stress tolerance and that this process was also involved in regulating the flowering time of chrysanthemum through influencing the expression of genes related to the photoperiod pathway and the GA pathway. These results led us to speculate that the function of *BBX24* in plant signaling is to act as a fine regulator of plant growth and development (Yang et al. 2014). Therefore, the aim of the study reported here was to further our understanding of the regulatory mechanism of *CmBBX24* expression by investigating how plant hormones, light, and abiotic stresses affect the activity of the *CmBBX24* promoter and by identifying the role of the *CmBBX24* promoter in the regulation of *CmBBX24* in response to abiotic stresses. The data obtained suggest that the *CmBBX24* promoter is

functional, particularly under conditions of abiotic stresses and hormone induction. Coupled with ongoing *CmBBX24* intron analyses, our results provide a solid foundation for the future use of the *CmBBX24* promoter in molecular breeding as a new promoter of stress-resistance genes from chrysanthemum.

## Materials and methods

### Isolation of the *BBX24* promoter

Total genomic DNA was extracted from samples of leaves using sodium the dodecyl sulfate technique (Milligan 1998). The 5′-upstream region of *CmBBX24* was isolated from chrysanthemum genomic DNA using reverse transcription-PCR and the nested PCR technique. The PCR product from this reaction was then inserted into a pGEM T-Easy vector (Promega, Madison, WI) for validity sequencing.

Using the PLACE database of motifs found in plant *cis*-acting regulatory DNA elements and the PlantCARE database of plant promoters (Higo et al. 1999; Guo et al. 2010), we analyzed and annotated the *cis*-elements in the sequence of the *CmBBX24* promoter.

### Construction of the deletion derivatives of the *CmBBX24* promoter

To analyze the spatial and temporal expression pattern of the *CmBBX24* gene and detect the activity of the *CmBBX24* promoter, we conducted a promoter deletion analysis in which we constructed a series of binary vectors using promoter fragments of different lengths (D0, D1, D2, D3, D4).

The sense primers were:

D4 *Hin*dIII forward: 5′-ATAAAGCTTACCATTTCTTTC CTTGAAACC-3′
D3 *Hin*dIII forward: 5′-GGTAAGCTTGGATGGATTTAT GTTGTAGGTTG-3′
D2 *Hin*dIII forward: 5′-GACAAGCTTCAAAAGGATC CATAATAACAAATC-3′)
D1 *Hin*dIII forward: 5′-ATCAAGCTTGAAGATCGAGC CATTGCTATG-3′
D0 *Hin*dIII forward: 5′-GTAAAGCTTCAAACTTAAAT ACAATGGATGACC-3′

The antisense primer was:
PSTH *Xba*I reverse: 5′-ATTCTAGACTTCAACACACA ACTTCTTCACTC-3′

Each sense primer was coupled with the antisense primer to amplify the corresponding deletion fragment. Each amplified sequence was then digested by *Hin*dIII and *Xba*I and inserted into the binary vector pBI121 to replace the cauliflower mosaic virus 35S promoter upstream of the β-glucuronidase (GUS) gene.

### *Arabidopsis* transformation

The vectors mentioned above were introduced into *Agrobacterium tumefaciens* strain GV3101, then transformed into Arabidopsis using the flower dip method described by Clough and Bent (1998). The independent transformants were screened on Murashige and Skoog (1962) medium (MS medium) supplemented with 50 mg $l^{-1}$ kanamycin. The T2 plants were used in this work as described by Tong et al. (2009).

### Abiotic stresses, hormones and monochromatic light treatments

To assay the response of the *CmBBX24* promoter to hormones and abiotic stresses, we germinated T2 seeds onto MS medium containing 3 %(w/v) sucrose and 0.6 %(w/v) agar at 23 °C under fluorescent white light and a LD photoperiod (16/8 h; light/dark). For the abiotic stress treatments, 9-day-old plants were transferred onto MS medium supplemented with different concentrations of mannitol or transferred into a cold chamber maintained at 4 °C for 4 days. For the hormone treatments, 9-day-old plants were transferred onto MS medium containing 40, 80 or 120 μM GA ($GA_{4+7}$), or onto 100, 200, 300, or 400 μM ABA for 4 days. For the light treatments, 9-day-old plants were transferred onto fresh MS medium and placed into separate chambers with white light, red light, blue light, and far red light for 24 h. The seedlings grown on MS medium under normal conditions (16/8 h light/dark) were used as controls.

### GUS histochemical assay

For the histochemical GUS analysis, plants were incubated in GUS staining solution containing 75.5 mM sodium phosphate (pH 7.0), 0.1 % Triton X-100, 0.05 mM $K_3$/$K_4$ FeCN, 10 mM Na2-ethylenediaminetetraacetic acid (EDTA), 20 % methanol (v/v), and 50 μg $ml^{-1}$ 5-bromo-4-chloro-3-indolyl glucuronic acid at 37 °C for 10 h. The plants were then washed and cleared by using 70 % ethanol and subsequently used for imaging.

### GUS activity assay

The fluorometric analyses of GUS activity were performed according to the method described by Jefferson (1987) and subsequently modified by Gallagher (1992). Plants were harvested and immediately homogenized by grinding in

0.3 ml GUS extraction buffer containing: 50 mM sodium-phosphate (pH 7.0), 10 mM EDTA, 0.1 % sodium laurylsarcosine, 0.1 % Triton X-100, and 10 mM β-mercaptoethanol. Cells debris was removed by centrifugation (8000 rpm) at 4 °C for 10 min, and the supernatant was collected. A 40-µl aliquot of supernatant was then added to 160 µl 4-methylumbelliferyl β-D-glucuronide hydrate (MUG) and mixed gently, following which 100 µl of this mixture was added to 900 µl (0.2 M) $Na_2CO_3$ and used as the control ($T_0$). The remaining 100 µl was incubated at 37 °C for 15 min in a water bath and then added to 900 µl (0.2 M) $Na_2CO_3$ and used as the Treatment sample ($T_1$), for the sample analysis. GUS activity was analyzed in a Microfluor fluorometer (model 450; Dynatec Scientific Labs, El Paso, TX) with emission at 455 nm and excitation at 365 nm. The protein concentration (*P*) of each sample was determined according to the method of Bradford (1976).

### Hypocotyl experiments

For all monochromatic light assays, plates were first cold-treated (4 °C) for 3 days and then transferred to continuous white light for 8 h to induce uniform germination. The plates were then transferred for culture under different light conditions and incubated at 22 °C for 6 days. Blue, red, and far-red light were generated by light emission diodes at 470, 670, and 735 nm, respectively (Model E-30LED; Percival Scientific, Perry, IA). Fluorescence rates for blue and red light were measured with a radiometer (model LI-250; LI-COR Biosciences, Lincolin, NE), and for far-red light, we used an opto-meter (40A Opto-Meter; United Detector Technology, Santa Monica, CA). Then the hypocotyl length of seedlings was measured.

### Drought treatment

Arabidopsis plants were grown in a controlled culture room [21 ± 1 °C with 70 % relative humidity, 16/8 h (light/dark), 150 µmol $m^{-2}$ $s^{-1}$]. For the drought treatment, four plants were grown in a 7-cm-diameter pot filled with 100 g of substrate [mixture of nutritional soil and vermiculite (1:1, v/v)]. Plants were watered sufficiently until 10 days old and then water was withheld for 30 days. The plants were then re-watered regularly as a recovery process. The survival rates were recorded after a recovery time of 10 days.

### Electrolytic leakage

The electronic leakage (EL) of transgenic Arabidopsis plants which carried various fusion constructs and of WT Arabidopsis plants was measured as a percentage according to the method of Ishitani et al. (1998) and Guo et al. (2002).

### Statistical analysis

As the experiment was conducted under controlled environmental conditions, a completely randomized design (CRD) was used to analyze the data. The data measured for five independent single-copy transgenic lines and each experiment were replicated four times. The means were compared using the least significant difference method at the 5 % level of probability. The statistical analysis software Microsoft Excel 2007 (Microsoft Corp., Redwood, WA) and Statistix-9 (Analytical Software, Tallahassee, FL) were used for the analysis of data.

## Results

### Isolation and *cis*-element analysis of the *CmBBX24* promoter

We isolated about 2.7 kb of the genomic DNA sequence upstream of the *CmBBX24* gene, including 198 bp of the 5′ untranslated region of the mRNA and the 2577-bp promoter sequence (Fig. 1a).

Using the PLACE database (Higo et al. 1999), we analyzed and annotated a number of possible *cis*-elements in the upstream sequence of the *CmBBX24* promoter. A putative CAAT box was identified at −340/−336, and a TATA box, which is thought to be essential for gene transcription initiation in plants, was located at −26/−32 (Grace et al. 2004). We also found an initiator motif (Inr), 50-CTCACTCC-30, at the −45/−38 position (Fig. 1a).

We identified four homologous sequences (5′-RYCGAC/ACCGAGA-3′) of the *cis*-acting dehydration responsive element (DRE), which responds to drought (Xue 2002, 2003), ten homologous sequences of the *cis*-element MYC (5′-CANNTG-3′), which responds to abiotic stress signals (Chinnusamy et al. 2003, 2004; Hartmann et al. 2005; Oh et al. 2005; Agarwal et al. 2006), five homologous sequences (5′-WAACCA/YAACKG/CNGTTR-3′) of MYB, which responds to dehydration, ABA signals (Abe et al. 1997, 2003; Busk et al. 1997), and two *cis*-acting sequences (5′- CCGAC-3′) of the low temperature responsive element (LTRE), which responds to cold, drought, and ABA signals (Baker et al. 1994; Jiang et al. 1996). We also found ABRE (1), GARE (1), and CARE (1) *cis*-regulatory elements, which respond to hormone signals (Ogawa et al. 2003; Nakashima et al. 2006), as well as eight homologous sequences of GT-1 (5′-GRWAAW/GGTTAA-3′), one CCA1 (5′-AAMAATCT-3′) sequence, and three homologous sequences of the T-box (5′-ACTTTG-3′) *cis* element, which are light responsive *cis*-elements (Chan et al. 2001) and so on (Fig. 1; Table 1). We also found seven TAAAG sequences that are guard cell-specific *cis*-elements (Plesch et al. 2001). The analysis showed that the possible

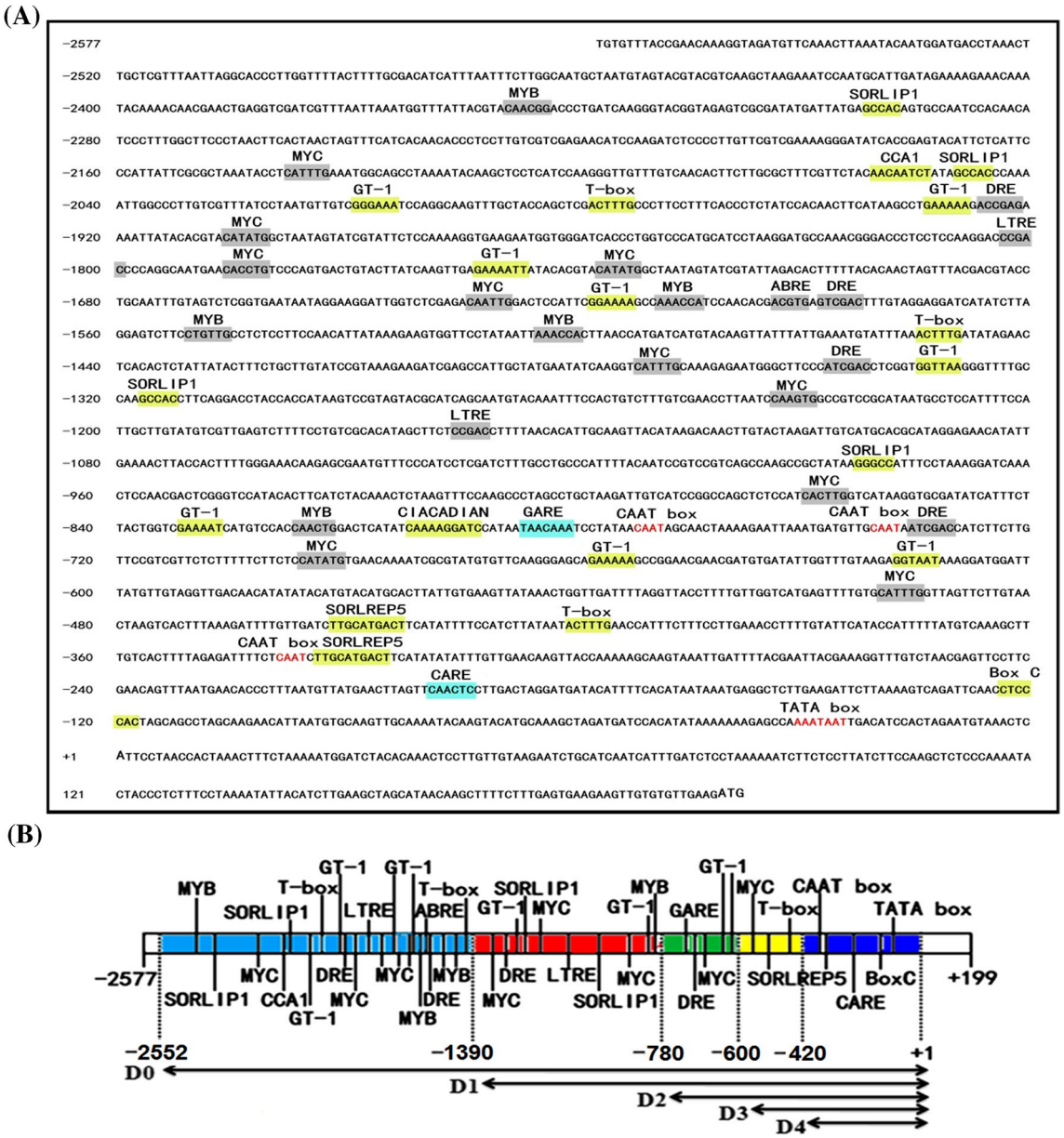


**Fig. 1** The isolated genomic DNA sequence and different segments with different *cis*-acting elements of the promoter of the B-box gene BBX24 from chrysanthemum (*CmBBX24*). **a** Pictorial representation of the *CmBBX24* promoter region with potential *cis*-element sites: promoter and putative *cis*-elements detected in the promoter region. *Numbering* The transcription start site was denoted *+1*. **b** Diagrammatic representation of the *CmBBX24* promoter regarding different segments with different *cis*-acting elements

*cis*-regulatory elements present in the *CmBBX24* promoter are mainly environment- or hormone-responsive motifs. Therefore, we considered that *CmBBX24* promoter might be an inducible promoter and be regulated by multiple abiotic factors and hormones.

## Analysis of *CmBBX24* promoter activity

To gain insight into the spatial and temporal expression pattern of the *CmBBX24* gene, we constructed a binary vector in which the GUS reporter gene was expressed under the control of the *CmBBX24* promoter and introduced the vector into the Arabidopsis genome by *Agrobacterium*-mediated transformation. We then detected GUS activity in the transgenic Arabidopsis plants throughout the whole life cycle at different growth and developmental stages by means of histochemical staining (Fig. 2). During vegetative growth of pBBX24::GUS transgenic plants, strong GUS activity was detected in the transition zones between the hypocotyls and roots and in the cotyledons and leaves,

**Table 1** *Cis*-regulatory elements identified in the *CmBBX24* promoter and their functions

| Factor or *cis*-regulatory element | *Cis*-regulatory element core sequence | No. of *cis*-elements | Description |
|---|---|---|---|
| DRE | RYCGAC/ACCGAGA | 4 | Dehydration-responsive element |
| MYC | CANNTG | 10 | Responds to abiotic stress signals |
| MYB | WAACCA/YAACKG/CNGTTR | 5 | Responds to dehydration and ABA signals |
| ABRE | ACGTG | 1 | ABA-responsive element-like |
| GARE | TAACAAR | 1 | GA-responsive element |
| CARE | CAACTC | 1 | CAACTC regulatory element/responds to GA signals |
| LTRE | CCGAC | 2 | Low temperature-responsive element/responds to cold, drought and ABA signals |
| GT-1 | GRWAAW/GGTTAA | 8 | Light-regulated transcription binding site |
| Box C | CTCCCAC | 1 | Responds to light signals |
| T-box | ACTTTG | 3 | Responds to light signals |
| CCA1 | AAMAATCT | 1 | CCA1-binding site/related to regulation by phytochromes |
| SORLIP1 | GCCAC | 4 | PhyA-induced motifs |
| SORLREP5 | TTGCATGACT | 2 | PhyA-repressed motifs |
| Guard cell-specific | TAAAG | 7 | Guard cell-specific *cis*-element |

*CmBBX24*, B-box gene *BBX24* from chrysantheumum; GA, gibberellic acid; ABA, abscisic acid; phyA, phytochrome A

especially in vascular tissues (Fig. 2n–r). Non-uniform staining was detected in the roots. Compared to GUS activity in young plants, GUS activity became weaker as the plant grew (Fig. 2a–g). However, during reproductive growth, GUS activity became stronger in flowers as they developed. At the early stages of flower development, GUS activity was detected in the sepals, petals, and stigma but not in stamens. With the opening of flowers, GUS activity was also clearly detectable in the stamens (Fig. 2i–m). As we found seven guard cell-specific *cis*-elements in the promoter of the *CmBBX24* gene, we also checked—and found—GUS activity in the guard cells (Fig. 2h).

## GUS activity of the different segments of the *CmBBX24* promoter in Arabidopsis

To understand the contribution of different regions in the *CmBBX24* promoter to its activity, we generated the D0 (−2552; full-length *CmBBX24* promoter), D1 (−1390), D2 (−780), D3 (−600), and D4 (−420) constructs. All constructs harbored *GUS*, but in each construct *GUS* was driven by a different deletion fragment of the promoter, as generated by PCR using specific primers based on the *cis*-element analysis (Fig. 3a). The different segments of the *CmBBX24* promoter with different *cis*-acting elements (D0–D4) are as shown in Fig. 1b. All five constructs were separately transferred into Arabidopsis by *Agrobacterium*-mediated gene transformation, following which we determined the GUS activity of the different *CmBBX24* promoter segments in leaves (Fig. 3b) and roots (Fig. 3c) of the five transgenic Arabidopsis lines by mean of the fluorometric assay. As shown in Fig. 3b, c, all constructs were sufficiently active to drive GUS expression, but GUS activity in leaves was more distinct than that in roots.

In leaves and roots, deletion of the 1162-bp fragment between −2552 (D0) and −1390 (D1) significantly reduced promoter activity, while no significant decrease in promoter activity was observed with further deletions from D1 to D2, D2 to D3, and D3 to D4. These results show that in the leaves and roots the basic *cis*-elements mainly exist between fragments D0 and D1.

At the same time, to gain further insight into the organ-specific expression profiles of the five deletion segments of the *CmBBX24* promoter (D0, D1, D2, D3, D4), we investigated GUS activity in the cotyledons, leaves, hypocotyls, roots, and flowers of corresponding transgenic seedlings using histochemical staining. As shown in Fig. 4, GUS activity in the cotyledons and leaves was stronger than in the hypocotyls, roots, and flowers in D0 (−2552) transgenic Arabidopsis. In hypocotyls and roots, deletion of the 1162-bp fragment between −2552 (D0) and −1390 (D1) resulted in nearly complete disappearance of GUS activity, while a gradual decrease in GUS activity with the deletion of the *CmBBX24* promoter was found in cotyledons, leaves, and flowers. The deletion of the fragment from −1390 (D1) to −780 (D2) and from −780 (D2) to −600 (D3) resulted in a sharp decrease in GUS activity in the cotyledons and leaves. In flowers, deletion of the fragment from −780

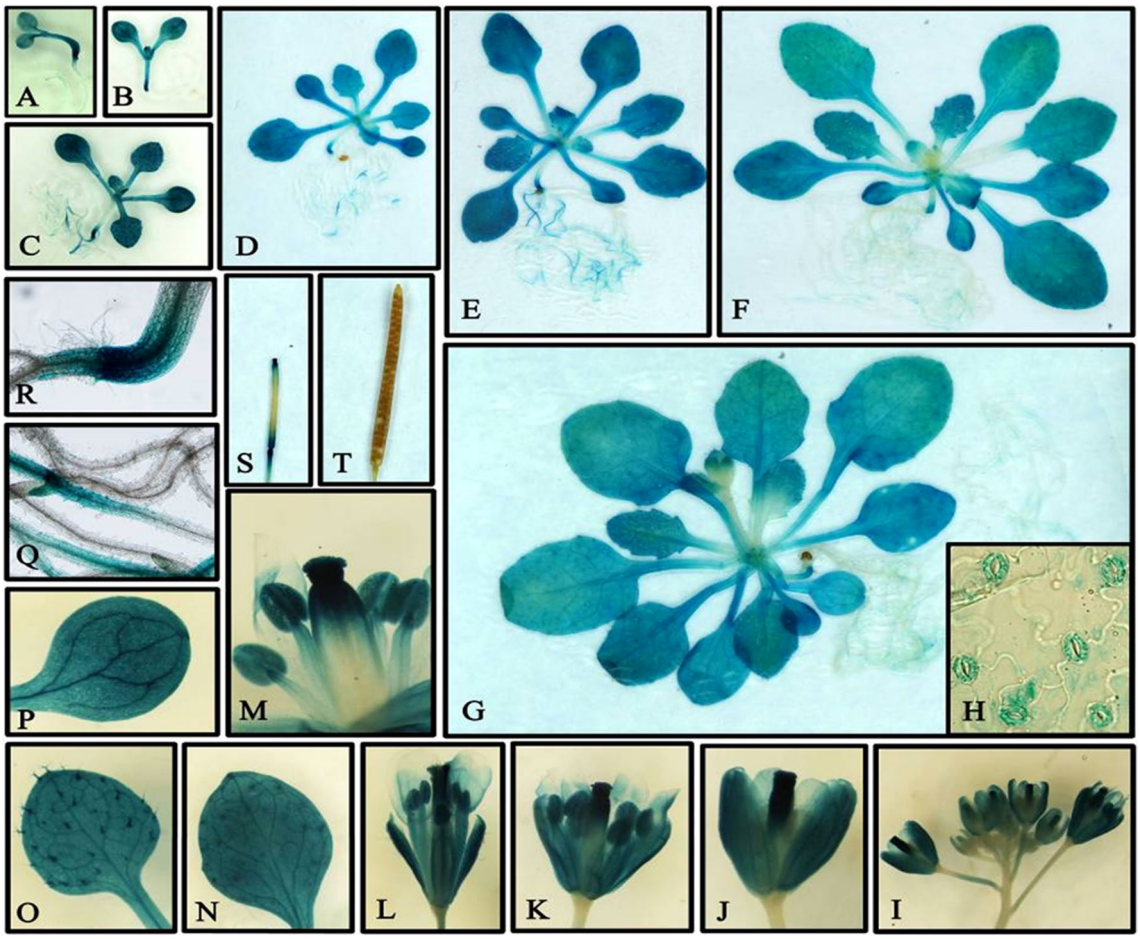


**Fig. 2** Analysis of β-glucuronidase (GUS) activity of the *CmBBX24* gene. **a–g** 3-, 6-, 9-, 12-, 15-, 18-, and 21-day-old plants, **h** stoma, **i** inflorescence, **j** unopened flower, **k** initial flower opening stage, **l** later stage of an opened flower; **m** stamen and stigma in detail, **n** old leaves, **o** young leaves, **p** cotyledons, **q** roots, **r** transition zones between hypocotyls and roots, **s** immature silique, **t** mature silique

(D2) to −600 (D3) also resulted in a sharp decrease in GUS activity.

These results suggest that most of the positive *cis*-regulatory elements are present in the region −2552 (D0) to −600 (D3), but that differences do exist in different organs. In hypocotyls and roots, the positive *cis*-elements are mainly present in the region −2552 (D0) and −1390 (D1). In flowers, positive *cis*-elements are present in the region −780 (D2) to −600 (D3), while in the cotyledons and leaves, they are diffused all around the region −2552 (D0) to −600 (D3).

### Effects of abiotic stresses on the activity of the different *CmBBX24* promoter segments

Our PLACE database (Higo et al. 1999) analysis revealed the presence of abiotic stress-related *cis*-elements, such as DRE, MYC, MYB, and LTRE, in the *CmBBX24* promoter sequence (Fig. 1a). DRE is a dehydration-responsive element (Xue 2002, 2003), MYC responds to abiotic stress signals (Chinnusamy et al. 2003, 2004; Hartmann et al. 2005; Oh et al. 2005; Agarwal et al. 2006), MYB responds to dehydration and ABA signals (Abe et al. 1997, 2003; Busk et al. 1997), and LTRE is a low temperature-responsive element which responds to cold, drought, and ABA signals (Baker et al. 1994; Jiang et al. 1996). Here, we investigated the subtle impact of mannitol and low temperature stresses on the activity of the *CmBBX24* promoter segments by means of the fluorometric assay for GUS activity.

Transgenic Arabidopsis plants containing the full-length *CmBBX24* promoter (D0) were initially subjected to 4 °C for 3 days to break the dormancy and then grown on MS medium for 9 days, following which they were transferred onto MS medium supplemented with 50, 100, 150, or 200 mM mannitol, or to just MS medium as a control. After 4 days of treatment, the plants were harvested and divided

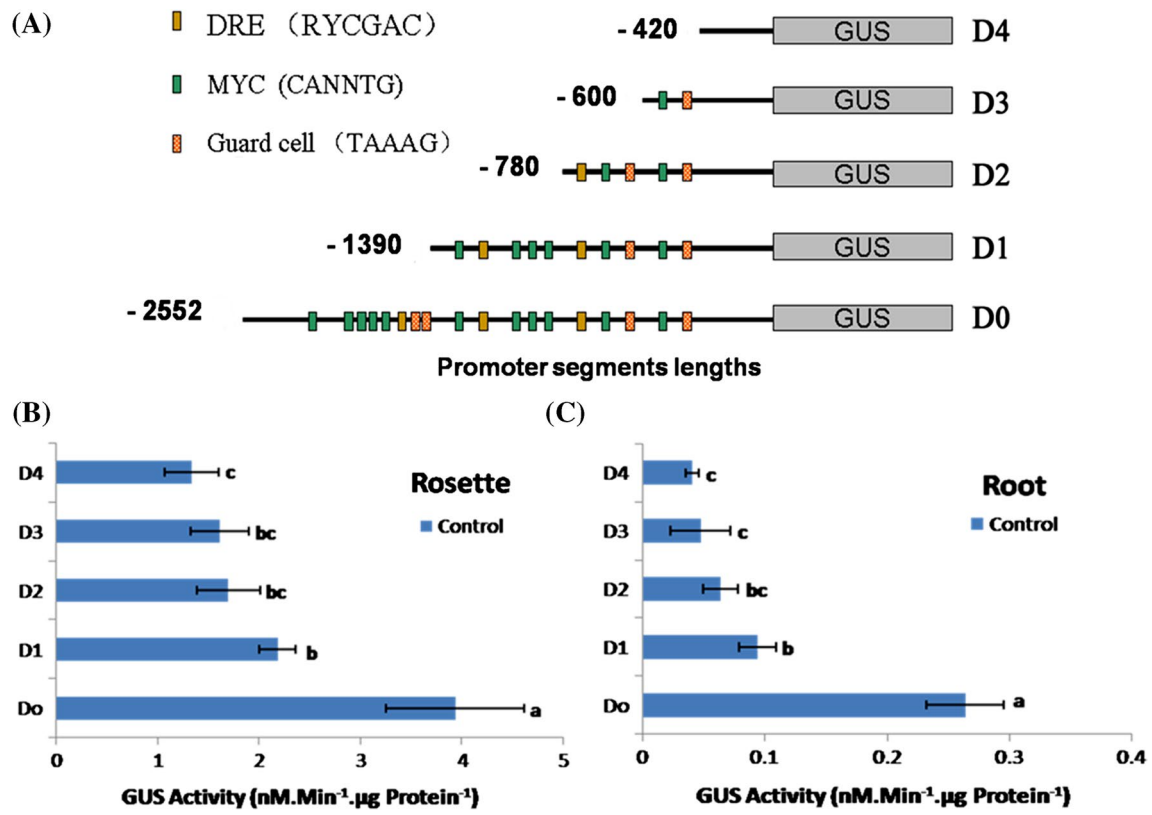


**Fig. 3** Assays of GUS expression driven by the complete and differently truncated *CmBBX24* promoters. **a** Schematic diagrams of the *CmBBX24* promoter deletions (*D0–D4*; see text for a full description) used to analyze the activity of different regions of the *CmBBX24* promoter. *Numbers to the left of the constructs* 5′ end of each deletion. All *CmBBX24* promoter deletions were fused to a GUS reporter gene. **b** GUS assays in leaves of transgenic Arabidopsis plants, **c** GUS assays in roots of transgenic Arabidopsis plants. The transgenic plants carrying the *CmBBX24* promoter with different deletions were grown on MS medium for 9 days, then transferred to fresh MS media for a further 4 days of growth, at which time they were harvested and used for the fluorometric assays. Data are presented as the mean (*bars*) and standard deviation (SD, *whiskers*) of GUS activity of four replicates. *Means followed by different lowercase letters* are significantly different at the 5 % level of significance

into rosette leaves and roots to determine the GUS activity in both parts separately. As shown in Fig. 5a, the promoter activity was highly induced by mannitol, with maximum activity appearing with at 50 mM mannitol. In a subsequent experiment, the transgenic Arabidopsis plants containing different segments (D0, D1, D2, D3 and D4) of *CmBBX24* promoter were transplanted to 50 mM mannitol, or to just MS as a control. All transgenic lines (D0, D1, D2, D3 and D4) containing the different promoter segments showed a clear response to mannitol in leaves compared with control, but the response to mannitol weakened gradually from about fourfold (D0) to twofold (D4) (Fig. 5b). In roots, the activity of all promoter segments was apparently not induced by mannitol (Fig. 5c).

Promoter activity was induced significantly by the low temperature treatment (4 °C), but weakened gradually with each fragment deletion in leaves; the response to low temperature ultimately disappeared with the deletion of the D3 to D4 segment (Fig. 5d). In roots, the activity was inhibited significantly with the deletion of fragments, from D0 to D1 and from D1 to D2; no significant inhibition was observed with further deletion of fragments (Fig. 5e).

The results indicate that mannitol and low temperature enhanced promoter activity in the leaves (Fig. 5b, d). The basic *cis*-elements responding to mannitol treatment were found to be uniformly scattered in the promoter, and those responding to low temperature were found mainly in the D3 to D4 region. In roots, none of the *cis*-elements in the promoter responded to the mannitol treatment (Fig. 5c), but low temperature inhibited promoter activity. We therefore propose that promoter activity mainly depends on the *cis*-elements in the region of D1 to D2 (Fig. 5e). Taking these results into accunt (Figs. 1b, 3a, 5b), we speculate that the main elements responding to mannitol, such as MYC, MYB, and DRE, may be uniformly scattered throughout the promoter and that other novel *cis*-elements

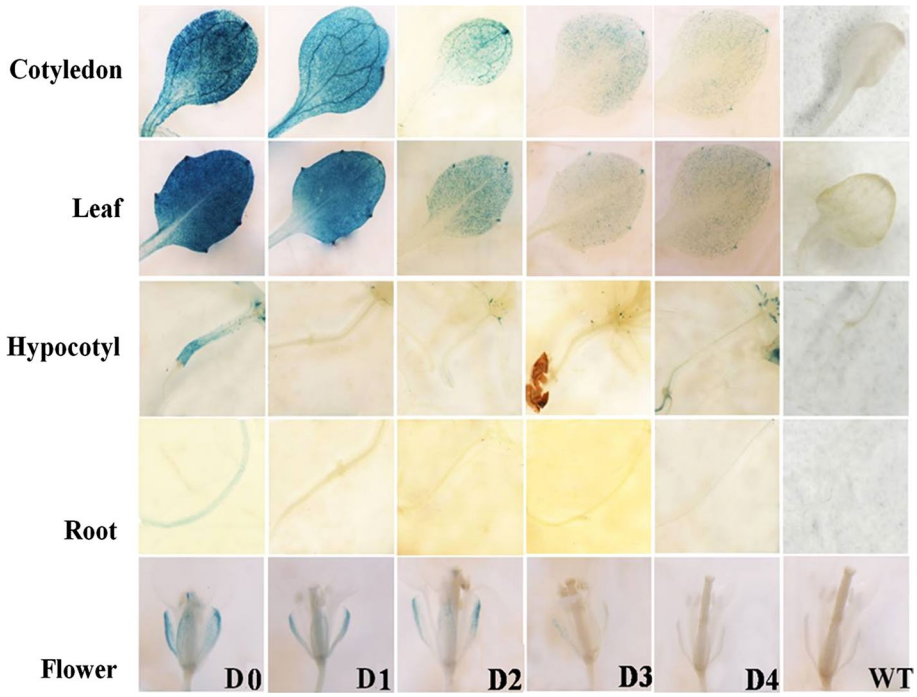


**Fig. 4** Histochemical location analysis of tissue-specific regulation of GUS gene expression in different plant organs of transgenic Arabidopsis plants driven by the different deletion segments (*D0–D4*) of the *CmBBX24* promoter. Cotyledons, leaves, hypocotyls, roots, and flowers of transgenic plants were stained for the detection of GUS activity. *WT* Wild type

may also be present in the D4 promoter. In leaves, it is possible that the MYC element in the D3 to D4 region is the main *cis*-acting element responding to low temperature in the promoter.

### Effects of hormones on the activity of the different *CmBBX24* promoter segments

The results of the experiments reported above led us to investigate the effect of GA and ABA on the activity of the *CmBBX24* promoter segments. Transgenic Arabidopsis seeds were cultured onto MS media and subjected to 4 °C for 3 days to break seed dormancy and then transferred to LD growing conditions. Transgenic 9-day-old Arabidopsis seedlings containing the full-length *CmBBX24* promoter (D0) were then transplanted onto MS media supplemented with different concentrations of $GA_{4+7}$ (40, 80, 120 μM) and ABA (100, 200, 300, 400 μM) for 4 days, following which GUS activity was measured.

In the case of ABA, all concentrations tested were sufficient to significantly enhance GUS activity, except for 100 μM. The highest response was recorded at 400 μM ABA, followed by 300 μM and 200 μM, respectively (Fig. 6a). In a subsequent experiment, we used 400 μM of ABA to assess distinct GUS activity for each of the five *CmBBX24* promoter segments. In rosette leaves, all of the five promoter segments clearly responded to ABA, but GUS activity induced by ABA was lower (onefold) in the D1 segment than in the D2 segment (twofold) (Fig. 6b). In roots, no significant response was recorded for D0, but other promoter segments showed an obvious response to ABA (Fig. 6c).

Similarly to the ABA treatment experiments, for the GA experiments transgenic Arabidopsis (9-day old) plants containing the full-length promoter (D0) were transplanted onto MS media supplemented with different concentrations of $GA_{4+7}$. GUS activity was enhanced significantly with increasing concentrations of $GA_{4+7}$ from 40 to 80 μM and then from 80 to 120 μM. The highest response was recorded at 120 μM $GA_{4+7}$. In contrast, there was no significant increase in GUS activity in plants exposed to 40 μM GA compared to the control (Fig. 6d).

Transgenic Arabidopsis (9-day-old) plants containing the different promoter segments were then transplanted onto MS supplemented with 120 μM $GA_{4+7}$ for 4 days and GUS activity analyzed. In leaves, all the promoter segments showed a response to GA, with the full-length promoter segment (D0) showing the most prominent activity; GUS activity significantly declined with the deletion of the 1162-bp deletion segement from D0 to D1 (Fig. 6e). In comparison, in roots, transgenic plants with the D0 and D2 segments responded significantly to $GA_{4+7}$, and plants with the other deletions showed no significant response (Fig. 6f).

These results indicate that 400 μM ABA and 120 μM GA enhanced the activity of all five promoter segments in the leaves (Fig. 6b, e). We speculate that the elements

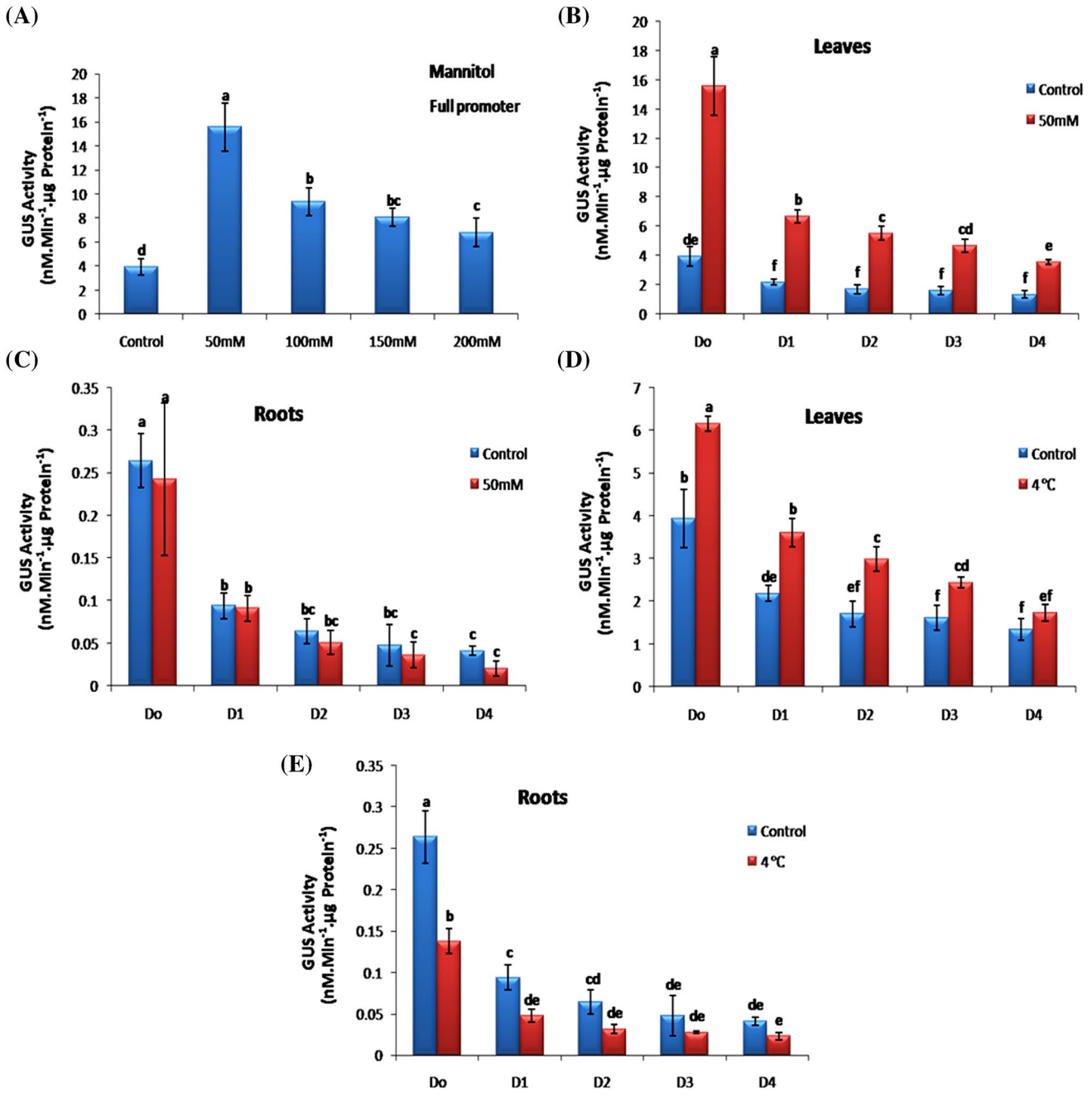


**Fig. 5** Quantification of the relative GUS activity in the leaves and roots of transgenic Arabidopsis plants containing the various deletion segments of the *CmBBX24* promoter (*D0–D4*) in response to the abiotic stresses of mannitol (50, 100, 150, or 200 mM) and cold (4 °C). GUS activity was measured immediately after treatment (4 days) in 9-day-old plants. **a** GUS assays of transgenic *Arabidopsis* plants containing the full promoter (*D0*) segment in response to the different concentrations of mannitol. **b**, **c** GUS assays of leaves (**b**) and roots (**c**) of transgenic *Arabidopsis* plants containing different *CmBBX24* promoter segments in response to 50 mM Mannitol. **d**, **e** GUS assays of leaves (**d**) and roots (**e**) of transgenic Arabidopsis plants in response to the cold stress (4 °C treatment, 4 days) treatment. Transgenic *Arabidopsis* plants without any treatment were used as the control. Data are presented as the mean (*bars*) and SD (*whiskers*) from five independent single-copy transgenic lines, with four replicates. *Means followed by different lowercase letters* are significantly different at the 5 % level of significance

mainly responding to ABA and GA may be uniformly scattered throughout the promoter and that ABA and GA response repressors without very strong inhibiting ability may be located in the segment from D1 to D2. Based on the prediction analysis of promoter *cis*-elements (Fig. 1), we did not find ABA-related *cis*-elements in the D4 segment,

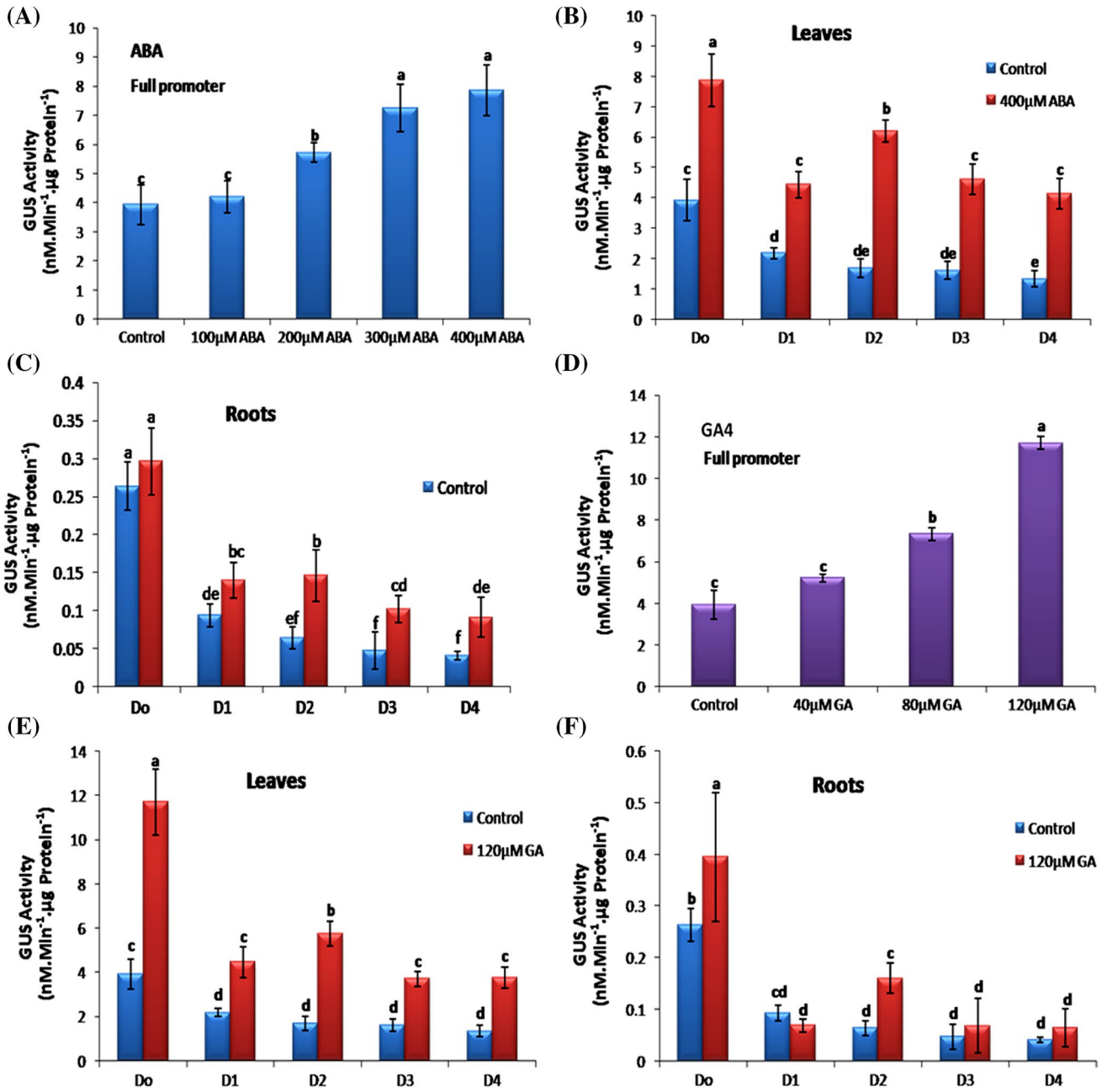


**Fig. 6** Quantification of relative GUS activity of various *CmBBX24* promoter segments in transgenic Arabidopsis plants subjected to treatment with gibberellic acid ($GA_{4+7}$) and abscisic acid (*ABA*). GUS activity was measured immediately after treatment in 9-day-old plants carrying various deletions of the *CmBBX24* promoter (*D0–D4*) transferred onto fresh MS media containing 100, 200, 300, and 400 µM ABA and 40, 80, and 120 µM $GA_{4+7}$, at 23 °C for 4 days. Transgenic Arabidopsis plants without any treatment were used as control. **a** GUS assays of transgenic Arabidopsis plants containing the full length promoter (*D0*) in response to different concentrations of ABA. **b**, **c** GUS assays in the leaves (**b**) and roots (**c**) of transgenic Arabidopsis plants in response to the 400 µM ABA treatment. **d** GUS assays of transgenic Arabidopsis seedlings containing the full promoter (*D0*) in response to different concentrations of $GA_{4+7}$. **e**, **f** GUS assays of the rosette (**e**) and roots (**f**) of transgenic Arabidopsis plants treated with $GA_{4+7}$. Data are presented as the mean (*bars*) and SD (*whiskers*) from five independent single-copy transgenic lines, and each experiment was replicated four times. *Means followed by different lowercase letters bars* are significantly different at the 5 % level of significance

leading us to suggest that ABA signaling is also independent of *BBX24* action to some extent. In addition, the concentration of the hormones tested also affected promoter activity, with increasing concentrations of $GA_{4+7}$ and ABA, respectively, increasing the activity of the promoter in each treatment (Fig. 6).

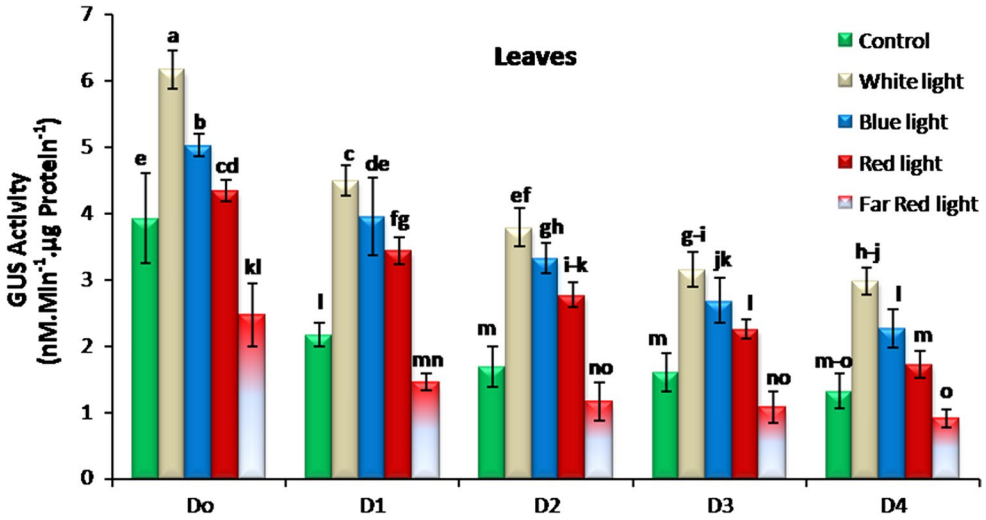


**Fig. 7** Quantification of relative GUS activity of various *CmBBX24* promoter segments under monochromatic light. GUS activity was measured immediately after treatment completion in the rosette leaves of 9-day-old plants transferred onto fresh MS media and then to four chambers illuminated with white light, far-red light, red light, and blue light, respectively, for 24 h. Transgenic Arabidopsis plants grown under normal conditions (16/8 h, light/dark) were used as controls. Data are presented as the mean (*bars*) and SD (*whiskers*) from five independent single-copy transgenic lines, and each experiment was replicated four times. *Means followed by different lowercase letters bars* are significantly different at the 5 % level of significance

### Effects of monochromatic light on activities of *CmBBX24* promoter segments

To understand the contribution of different parts of the light spectrum to the activity of the *CmBBX24* promoter, we transplanted 9-day-old transgenic Arabidopsis plants harboring one of the five regions of promoter to fresh MS media and treated them with monochromatic light, including red light, blue light, far-red light, and also white light, for 24 h. Plants grown under normal growing conditions (16/8 h, light/dark) were used as controls. Whole transgenic plants were divided into two parts (rosette leaves and roots), and the fluorometric assay was used to check GUS activity. As shown in Fig. 7 and Electronic Supplementary Material (ESM) Fig. S1, GUS activity was higher when the plants were exposed to white light, followed by blue light, red light and far-red light in the order of decreasing GUS activity both in leaves (Fig. 7) and in roots (ESM Fig. S1). Interestingly, compared to the control, white light, blue light, and red light enhanced promoter activity, but far-red light inhibited it.

In leaves, the apparent significant response to white and blue light was recorded in all deletion segments. The response to red light was obvious in D0, D1, D2, and D3 segments, but disappeared with the deletion of D3 to D4. Meanwhile, the inhibition by far red light mainly depend on the segment of D3 to D4 (Fig. 7a).

In the case of roots the obvious responses to white light disappeared with the deletion of D3 to D4 segment, the response to blue light disappeared with the deletion of D1 to D2, and the response to red light and the inhibition by far red light disappeared with the deletion of D0 to D1 (Figure S1). In roots, the *cis*-elements response to blue light was mainly located in the D1 to D2, and may be the GT-1 *cis*-element. The element response to red and far red lights mainly located in the D0 to D1, with the GT-1 and T-box elements.

These results concludes that all segments were enough to enhance promoter activity in response to white and blue light in leaves, while the D4 segment cannot respond to red and far red lights, so we speculate that the Box-C in the D4 segment may play a role in blue light response, and the T-box in the D3 to D4 segment may play a role in red and far red light response. These results demonstrate that monochromatic blue light has a major part in GUS activity of promoter in response to light.

### Effects of light conditions and abiotic stresses on the development of *CmBBX24* overexpression in Arabidopsis

Our evaluation of *CmBBX24* expression in chrysanthemum revealed that abiotic stresses and monochromatic lights affected the expression level of *CmBBX24* (ESM Fig. S2).

We then investigated the growth of *CmBBX24*-OX transgenic Arabidopsis hypocotyls under the different light conditions, including continuous dark or white light conditions, different monochromatic light, and different daylengths with white light (Fig. 8a, b). In the continuous dark treatment, the hypocotyls were very long, and there were no

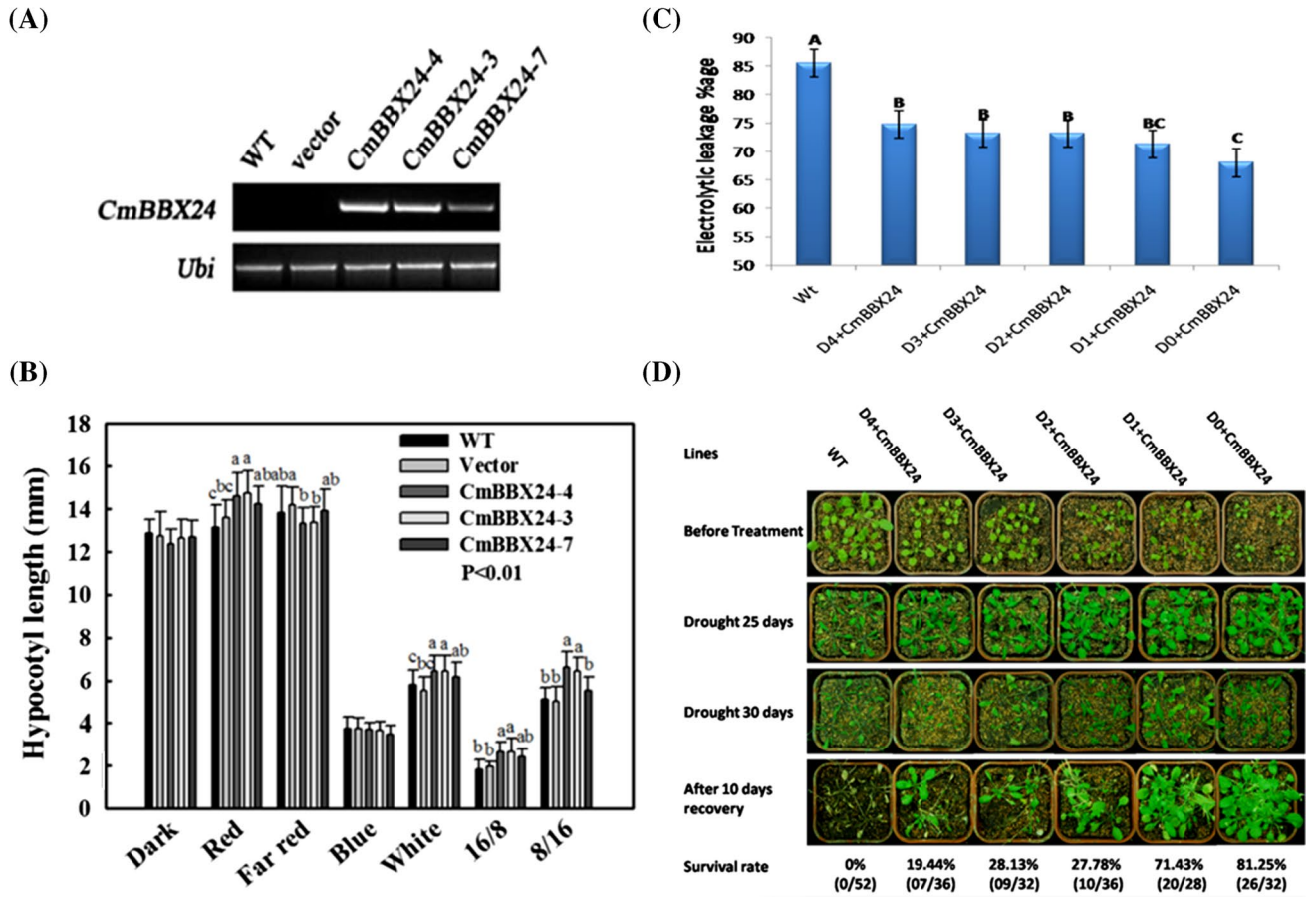


**Fig. 8** Hypocotyl length under various light conditions and abiotic stress tolerance of wild-type (*WT*), *CmBBX24*-vector-containing plants, and transgenic *CmBBX24* plants with different truncations of the *CmBBX24* promoter. **a** Expression determination of *CmBBX24* in overexpressed plants, **b** Arabidopsis seeds were germinated under continuous dark (*Dark*), red light (*Red*), far-red light (*Far red*), blue light (*Blue*), white light (*White*), and long day (*16/8*, white/dark) and short day (*8/16*, light/dark) conditions for 6 days, respectively. *Vector* Control plants,*CmBBX24-4* *CmBBX24*-3, *CmBBX24-7* three independent *CmBBX24* overexpressing lines. $P < 0.01$. **c** Electrolytic leakage of WT, *CmBBX24*-vector-containing plants, and transgenic *CmBBX24* Arabidopsis plants in response to cold temperature (−6 °C) treatments. Data are presented as the average (*bars*) and SD (*whiskers*) of three replicates. *Means with different lowercase letters bars* are significantly different at the 5 % level of significance. **d** WT, *CmBBX24*-vector-containing plants, and transgenic *CmBBX24* Arabidopsis plants were cultured in vitro, following which 10-day-old plants were transferred to soil and grown under long day conditions. The plants were watered to saturation during the first 10 days after transplantation, then water was withheld for 30 days, and finally the plants were watered again. Data on survival (%) and pictures were taken after 10 days of recovery

significant differences in length among transgenic and WT plants. In contrast, in the white light treatments, including continuous light treatment, LD, and SD treatments, shorter hypocotyls were detected, relative to the lengths of those in the continuous dark condition, with longer hypocotyls observed in the transgenic plants than in WT plants. In the monochromatic light experiments, elongation was detected in hypocotyls under red light and far-red light, with results similat to those in the dark treatment. Transgenic Arabidopsis plants had longer hypocotyls under red light and shorter hypocotyls under far-red light, and no obvious differences were detected among transgenic and WT plants under blue light (Fig. 8b).

These results suggest that in the light-dependent early morphogenesis process, *CmBBX24* negatively regulates red light signaling and acts as a positive regulator in far-red light signaling. Similar results were observed regarding the activity of the *CmBBX24* promoter to monochromatic light.

In order to identify the role of the *CmBBX24* promoter in *CmBBX24*-regulated tolerance to abiotic stresses in Arabidopsis, we linked different truncations of the *CmBBX24* promoter to GUS and to the open reading frame of *CmBBX24* and transformed these constructs into Arabidopsis plants. We observed that all of the *CmBBX24* promoter segments were sufficient to significantly enhance abiotic stress tolerance in transgenic Arabidopsis plants compared

to the WT Arabidopsis plants (Fig. 8c, d). To check the tolerance to cold temperature, we measured the EL percentage of transgenic and WT Arabidopsis plants due to damage caused by the freezing temperature. We found that the EL percentage of transgenic plants with any one of the promoter segments was significantly lower than that of the WT plants. We also observed that among the *CmBBX24* segments, the D1- and D2-pro*CmBBX24*::*CmBBX24* lines had a significantly lower EL percentage and that the other promoter segments (D2, D3, D4) had no significant effect (Fig. 8c).

We also observed that all of the promoter segments were sufficient to significantly enhance drought stress tolerance in transgenic Arabidopsis plants and further observed that the D0- and D1-pro*CmBBX24*::*CmBBX24* lines enhanced drought stress tolerance compared to the D2-, D3-, and D4-pro*CmBBX24*::*CmBBX24* transgenic and WT Arabidopsis plants (Fig. 8d).

These results indicate that *CmBBX24* is an inductive promoter and that *CmBBX24* plays an important role in regulating abiotic stress tolerance in transgenic Arabidopsis plants.

## Discussion

Genes can be expressed during different plant growth and developmental stages in different tissues with constitutive promoters (Guo et al. 2010). When the promoter is very active, the transformed genes driven by constitutive promoters can cause homology-dependent gene silencing (Vaucheret et al. 1998). The unique advantages of strong inductive promoters extracted from different plants make them powerful tools by which to improve plant resistance to abiotic stresses. Tissue-specific and inducible promoters are always preferred elements for transformation into plants and can be analyzed in abiotic stress experiments. To date, however, very few shuttle promoters have been discovered (Guo et al. 2010).

In our previous work, we isolated the full-length transcript of the *BBX24* gene from young leaves of chrysanthemum and found that the gene plays an important role in abiotic stress tolerance and the flowering process in chrysanthemum (Yang et al. 2014). In the present investigation, we studied the *CmBBX24* promoter and found it to be very active, playing an important role in response to abiotic stress, hormones, and light treatments. This promoter therefore has the potential to be a powerful tool for future use in the molecular breeding of ornamental plants.

Promoters are segments of DNA which induce gene transcription according to specific conditions. They are located in close proximity to a gene's transcription start site, in the upstream region on the same DNA strand. In our study, we used the PLACE and PlantCARE databases (Higo et al. 1999; Guo et al. 2010) and annotated a number of possible *cis*-elements in the upstream sequence of *CmBBX24*. We observed a putative CAAT-box at position −340/−336 and the TATA-box at position −26/−32 (Fig. 1a; Table 1), which is essential for gene transcription initiation in plants (Grace et al. 2004). We also found an initiator motif (5′-CTCACTCC-3′) at the −45/−38 position. This motif is rarely found in plants as the core motif for transcription initiation in promoters without a TATA-box (Nakamura et al. 2002), although it is frequently found in mammalians (Smale and Baltimore 1989). The identified *cis*-elements included abiotic stress-related elements, such as DRE, MYC, MYB, and LTRE, hormone signaling-related elements, such as ABRE, GARE, and CARE, light-responsive elements, such as GT-1, CCA1, and T-box, tissue-specific elements (e.g., guard cell elements), among others (Fig. 1a, b; Table 1).

We found four DRE, one ABRE, ten MYC, five MYB, and two LTRE *cis*-elements in the *CmBBX24* promoter (Table 1) which responded to different abiotic stresses, including drought and low temperature. As there appeared to be more MYB and MYC than DRE and LTRE *cis*-elements, the *CmBBX24* promoter may be induced more by the transcription factors MYB and MYC than by DRE and ABRE elements.

Once we had performed the PLACE analysis, we transformed the full-length (D0) promoter and four promoter segments of different lengths (D1, D2, D3 and D4) into Arabidopsis and subjected the T2 transgenic plants to GUS histochemical staining. We detected a sharp decrease in gene expression in the hypocotyls and roots with the deletion of the 1162-bp fragment from D0–D1, compared with the GUS activity detected in D0. In the leaf, GUS activity gradually decreased from D0 to D4. However, by removing −1162 bp from the 5′-end of the full-length (−2552 bp) *CmBBX24* promoter under normal conditions in the absence of stress induction, the activity significantly declined in the intron-mediated transcription in transgenic lines. In addition, no staining was recorded upon D0–D1 deletion from the 5′-end of D0 in roots, indicating that some positive-regulatory elements might exist in this fragment. In the leaf, GUS activity decreased from D0 to D2 and fell sharply in D3, indicating there were important elements present in the D0–D2 segment, as well as an an enhancer-like sequence (Fig. 4).

The fluorometric assay of GUS was used to observe *CmBBX24* promoter activity. In these experiments, transgenic Arabidopsis plants containing either the full-length (D0) promoter or one of four deletion segments of different length (D1, D2, D3 and D4) were exposed to low temperature and different mannitol concentration treatments. In leaves, almost all of the segments were sufficient

for enhancing the GUS activity in transgenic Arabidopsis plants as compared to control plants. Promoter activity was clearly present in rosette leaves. However, in the roots mannitol had no significant effect on promoter activity, and lower temperature suppressed promoter activity, as compared to control (Fig. 5). On the other hand, with increasing mannitol concentration, *CmBBX24* promoter activity declined gradually (Fig. 5a). All segments of the *CmBBX24* promoter were also induced by GA and ABA treatments, and a similar GUS activity response was observed in the $GA_{4+7}$ and ABA treatments. With increasing concentrations of ABA and $GA_{4+7}$, promoter activity was enhanced, and the activity of all promoter segments, except in roots in the $GA_{4+7}$ treatment, was higher in the rosette leaves than in the roots (Fig. 6). The deletion of the promoter region 1162 bp from D0 to D1 significantly reduced the GUS activity of the *CmBBX24* promoter, but further deletion of the 610 bp from D1 to D2 resulted in recovery in the GUS activity in response to ABA and $GA_{4+7}$ treatments. Based on these results we speculate that the 610-bp promoter region from D1 to D2 may contain some hormone response-suppressing elements which result in the reduction of promoter activity, but that the hormone-suppressing elements are not very strong so that while the activity of the D1 segment is reduced, there is no effect on the full-length (D0) promoter or the D2 segment. With further deletion of the 180-bp segment from D2 to D3, we observed once again a significant decline in promoter activity. However, our PLACE analysis did not reveal an ABA-related element in the D2, D3, or D4 segments and also did not identify GA-related elements in the D0 to D1 segment (1162 bp). Therefore, we speculate that ABA signaling is also independent of *CmBBX24* to some extent.

As in our previous work we found that *CmBBX24* acts as a negative regulator in phytochrome and light signaling (Yang et al. 2014). In the present investigation we examined *CmBBX24* promoter activity in response to white light, blue light, red light, far-red light, and monochromatic light. We observed that all segments were sufficient to induce GUS activity in leaves, with the exception of far-red light which inhibited promoter activity in both the leaves and roots. Here, the promoter activity was also higher in the rosette than roots. Promoter activity significantly declined with every deletion from D0 to D3, although the reduction was less with the deletion from D3 to D4 (Fig. 7), which suggests that the promoter region from D0 to D3 is rich in light-responsive elements.

In our experiments, activation of the *CmBBX24* promoter was induced by white light, monochromatic blue light, and red light, but inhibited by far-red light. Blue light plays a major part in the activity of the promoter in response to light. However, the phenotypes of *CmBBX24*-OX transgenic Arabidopsis lines under different light treatments are not completely consistent with the response to different light sources. Under the white light treatment, regardless of the treatment (continuous white light, LD, or SD), hypocotyls were longer in transgenic plants than in WT plants. In case of the monochromatic light, transgenic plants had longer hypocotyls under red light and shorter hypocotyls under far-red light treatment, which is consistent with the promoter activation results in the monochromatic light treatments. In the blue light treatment, there were no obvious differences detected between transgenic and WT plants. This result is not in line with the results of the *BBX24* gene analysis in Arabidopsis (Indorf et al. 2007). We speculate that there is a post-transcriptional blue light-dependent degradation of *CmBBX24* protein but that this degradation cannot resolve all of the protein induced by white light. Although a light-dependent accumulation of BBX proteins has been reported, but found that this accumulation was transient, not continuous (Indorf et al. 2007).

*Cis*-acting elements are important regulatory molecular components that play important roles in the transcriptional regulation processes of various dynamic networks of gene activities which control different biological processes, including abiotic stress response, hormone responses, and development processes. Transcriptome expression profiling experiments have led to the identification of different combinations of *cis*-acting elements in the promoter regions of various genes which respond to different stresses and hormones (Yamaguchi-Shinozaki and Shinozaki 1994, 2005; Guo et al. 2010). In our study, we investigated major *cis*-acting elements that are vital parts of gene expression in stress and hormone response elements by fluorometric analysis of the GUS activity of *CmBBX24* promoter segments. The *CmBBX24* promoter segment positively responded to mannitol, cold, GA, ABA, and monochromatic light.

To study the activity of the *CmBBX24* promoter under abiotic stresses and monochromatic light, we developed the *CmBBX24* gene linked to different truncations of *CmBBX24* promoter and transgenic Arabidopsis plants carrying *GUS* and observed that those *CmBBX24* promoter segments linked to the *CmBBX24* significantly enhanced abiotic stress tolerance (Fig. 8c, d). These results are supported by the results of previous studies, such as *DREB1A*/*CBF3* overexpression driven by the stress-inducible promoter (rd29A), which enhanced drought stress tolerance in transgenic wheat (Pellegrineschi et al. 2004). Similarly, the *CBF3*/*DREB1A* constitutive overexpression driven by the 35S promoter enhanced the drought and high salinity stress tolerance in transgenic rice (Oh et al. 2005).

The *CmBBX24*-overexpressing Arabidopsis plants responded to different monochromatic light sources. We also found that the promoter was able to drive *CmBBX24* adaptation to abiotic stresses of transgenic Arabidopsis plants (Fig. 8). Taking into account all of our results, we

speculate that *CmBBX24* is of prime importance and that the *CmBBX24* promoter is inductive under abiotic stress conditions. However, the potential use of the *CmBBX24* promoter in breeding programs should be demonstrated.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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