Genome-wide and expression analysis of B-box gene family in pepper

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Abstract
Background  BBX transcription factors are a kind of zinc finger transcription factors with one or two B-box domains, which particulate in plant growth, development and response to abiotic or biotic stress. The BBX family has been identified in Arabidopsis, rice, tomato and some other model plant genomes.

Results  Here, 24 CaBBX genes were identified in pepper (Capsicum annuum L.), and the phylogenetic analysis, structures, chromosomal location, gene expression patterns and subcellular localizations were also carried out to understand the evolution and function of CaBBX genes. All these CaBBXs were divided into five classes, and 20 of them distributed in 11 of 12 pepper chromosomes unevenly. Most duplication events occurred in subgroup I. Quantitative RT-PCR indicated that several CaBBX genes were induced by abiotic stress and hormones, some had tissue-specific expression profiles or differentially expressed at developmental stages. Most of CaBBX members were predicted to be nucleus-localized in consistent with the transient expression assay by onion inner epidermis of the three tested CaBBX members (CaBBX5, 6 and 20).

Conclusion  Several CaBBX genes were induced by abiotic stress and exogenous phytohormones, some expressed tissue-specific and variously at different developmental stage. The detected CaBBXs act as nucleus-localized transcription factors. Our data might be a foundation in the identification of CaBBX genes, and a further understanding of their biological function in future studies.

Keywords  BBX, Pepper, Phylogenetic relationships, Subcellular localizations, Gene expression patterns

Introduction
In plant, transcription factors (TFs) are a kind of proteins that play an important part in physiological and biochemical processes by regulating the downstream gene transcription. There are usually four main domains for TF structure construction, all of which required for the functional process: DNA binding site, transcription activation domain, oligomerization site, and nuclear localization signal [1]. Among them, the study of B-box (BBX) zinc finger family is a growing area in recent years. The BBX transcription factors in plants usually carry one or two B-box domains (CX2CX8CX7CX2CX4HX8H) in the N-terminal region, in which the conserved Cysteine (C) and Histidine (H) residues are predicted to be involved in protein-protein interactions [2]. The conserved B-box domains consisting of 40 amino acids was widely found in more than 1500 proteins of multicellular species and some unicellular eukaryotes [3]. Also, some plant BBX proteins are characterized containing an additional highly conserved CCT (CONSTANS, CO-like, and TIMING of CAB1) domain in the C-terminus [4, 5], which play an essential role in transcriptional regulation and nuclear transport [6, 7]. There are 32 BBXs found in
Arabidopsis, named AtBBX1 ~ 32 [2]. These AtBBX proteins were divided into five structure groups (Groups I ~ V) depending on the number and sequence features of the B-box domain or the presence of a CCT domain [2, 8, 9]. Both Group I (AtBBX1 ~ 6) and Group II (AtBBX7 ~ 13) proteins possess two B-box domains and one CCT domain, with some differences at consensus sequences of second B-box domain between Group I and II [8]. AtBBX14 to AtBBX17 belonging to Group III had a single B-box domain in association with a CCT domain; the AtBBX members of structure group IV (AtBBX18 ~ 25) contain two B-box domains without CCT domain; while Group V proteins (AtBBX26 to AtBBX32) only had a single B-box domain [2]. Additionally, the BBX TFs were identified in several other model plants in recent years, such as 30 members in rice, 29 in tomato, 64 in apple, 39 in pear, and so on [10 ~ 14]. And the BBX TFs identified in all these model plants were classified into five groups in the same cases with Arabidopsis BBX members.

In plants, B-box (BBX) proteins are well-known to be involved in plant development, especially light, circadian signaling and flowering. CO/AtBBX1 was the first discovered BBX protein, a core component which can promote flowering under long-day condition [15, 16]. Other two BBX proteins, BBX2 and BBX3 were investigated to be less influenced on flowering time, but over-expression of BBX2 gene showed a decreasing duration of two specific circadian rhythms in Arabidopsis [17]. BBX21 (also known as SALT TOLERANCE HOMOLOG 2) were identified as a regulator of several ABA INSENSITIVE (ABI) genes and directly activates ELONGATED HYPOCHOTYL 5 (HY5) in ABA control of seed germination, which is targeted by COP1 for 26S proteasome-mediated degradation in Arabidopsis [18 ~ 20]. AtBBX28 was found that interact with both HY5 and COP1 at its C-terminal portion resulting in negatively regulates photomorphogenic development [21]. Besides, HY5 negatively regulated BBX30 and BBX31 by directly binding to the G-box cis-element present in their promoters, negatively regulate photomorphogenesis in Arabidopsis [22]. BBX4 is a key component involved in the phyB (Phytochrome B)-PIF3 (PHYTOCHROME INTERACTING FACTOR 3) regulatory module. phyB directly interacts with BBX4 and positively controls the level of BBX4 protein in red light. And BBX4 repressed the transcriptional activation activity of PIF3 by directly interacting with PIF3, thereby promoting photomorphogenesis [23]. And BBX11-BBX21-HY5 can positively regulate photomorphogenesis in the response to light during normal development [24]. Besides, several BBX are identified to be involved in flowering by positively and negatively regulating the CO (CONSTANS) and FT (Flowering Locus T) genes expression [25 ~ 29]. In rice, OsCO3 possessing a single B-box and CCT domain functions as a negative FT-like genes regulator which delays flowering time under SD (short day) conditions [30]. OsCLO4 showed a represses flowering under SD and LD (long day) conditions [31]. And Hd1 (OsBBX18) containing two B-box motifs and one additional CCT domain, promote flowering under SD conditions and inhibit under LD conditions [4, 32]. Nevertheless, several BBXs in other plants, such as barely (Hordeum vulgare), beetroot (Beta vulgaris), chrysanthemum (Chrysanthemum morifolium), and grape (Vitis vinifera), also play an important role in regulation of flowering [4, 30, 33].

In addition, BBX family genes have shown their roles in mitigating abiotic stresses. The salt tolerance protein (STO, AtBBX24) was first identified to trigger the salt tolerance activities in yeast cells [34], which can enhance Arabidopsis root growth under salt stress treatment [35]. STO negatively regulated a wide range of stress-related genes [36], which can also interact with CLONE EIGHTY-ONE/RADICAL INDUCED CELL DEATH1 (CEO/RCD1) [37, 38]. AtBBX18 was detected to be a negative regulator both in photomorphogenesis and heat tolerance in Arabidopsis [39]. In rice and tomato, most promoters of the OsBBX and SlBBX genes contain at least one stress-responsive cis-element (ARE, Wbox, GC-motif, Box-W1, HSE, and MBS). Through the expression analysis under biotic or abiotic stress, the expression levels of most of BBX family members in the rice and tomato were significantly changed under most treatments, indicating that these genes were induced by biotic or abiotic stress [10, 11]. In Chrysanthemum, CmBBX24 not only play a part in delaying flowering time, but also enhance cold and drought tolerance in plant [33]. Besides, overexpression of VvBBX32 can increase cold tolerance in transgenic Arabidopsis plants [40]. Recently, an apple B-box protein BBX37 was identified that regulates jasmonic acid mediated cold tolerance through the JAZ-BBX18-ICE1-CBF pathway [41]. Although the studies of BBX transcription factors are increasing rapidly in previous years, there are few studies about BBX genes in pepper [42]. And the whole genome sequencing of Capsicum annuum L. makes it possible for analyzing deeply in the BBX gene family of pepper [43, 44].

Pepper (Capsicum annuum L.) is a dominant vegetable species belonging to Solanaceae family cultivated all over the world. And in recent one or two years, it has overtaken tomato, with the cultivated area in first place in the world. However, the vegetative and reproductive growth of pepper was negatively affected by biotic and abiotic stresses such as salt, cold, heat, drought, diseases and insect pests. And BBX are thought to play important roles in plant abiotic and biotic stress responses, thus the study of CaBBX TFs in these molecular mechanisms is
necessary to determine the biological processes involved in the multiple regulatory of abiotic tolerance. In this study, 24 CaBBX members were identified in pepper. And we also performed the gene structure, phylogenetic relationships, chromosome localization, subcellular localizations and their expression patterns under various abiotic stresses and hormones treatments in pepper.

**Materials and methods**

**Identification of BBX family genes in pepper**

We firstly obtained the conserved B-box domain (PF00643) based on a Hidden Markov Model (HMM) from the Pfam database (Pfam 32.0, [http://pfam.xfam.org/](http://pfam.xfam.org/)). Then the HMM profile of the B-box domain was utilized to do BLASTP search by using HMMER 3.2 in pepper genome databases with an expected value (e-value) cut-off of 0.01 [43, 44]. Afterwards, the putative CaBBX proteins were confirmed for the presence of the B-box domain by the SMART ([http://smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)) and Pfam ([http://pfam.xfam.org/](http://pfam.xfam.org/)) searches and InterProscan ([http://www.ebi.ac.uk/interpro/search/sequence-search](http://www.ebi.ac.uk/interpro/search/sequence-search)) programs. In addition, the isoelectric point (pI) and molecular weight (kDa) of the obtained CaBBX proteins were determined by using the ExPASy proteomics server ([https://web.expasy.org/](https://web.expasy.org/)) [45].

**Phylogenetic analysis and sequence alignment**

The BBX sequences of tomato were obtained from the NCBI database ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). Multiple sequence alignments of CaBBX proteins were carried out with the ClustalX program (Version 2.1) [46]. The p-distance-based phylogenetic tree was constructed with the neighbor-joining algorithm in MEGA (version 7.0) with a bootstrap value of 1000 [47].

**Domains, motif structure and gene structure analysis**

Domains were identified with Conserved Domain Database (CDD) in NCBI ([https://www.ncbi.nlm.nih.gov/cdd](https://www.ncbi.nlm.nih.gov/cdd)). MEME Suite was used to determine all motifs in the CaBBX protein sequences [48]. Analysis was performed using the following parameters: number of repetitions, if any; optimum width of the motif, 6–50; and maximum number of motifs, 8. And the intron and exon were determined by CDS and genomic information in pepper Genome Database ([http://pepperhub.hzau.edu.cn/](http://pepperhub.hzau.edu.cn/)). All these structures were visualized by TBtools [49].

**Chromosomal location and duplication analysis of CaBBXs**

The identified CaBBX gene annotations and their chromosomal locations were retrieved from genome annotations downloaded from the Pepper Genome Database ([http://pepperhub.hzau.edu.cn/](http://pepperhub.hzau.edu.cn/)) according to the gene ID. The exact location of genes on chromosomes was drawn by using TBtools. Duplication analysis was also constructed by using TBtools [49].

**Plant materials, growth condition, hormone and stress treatments to plants**

Pepper seeds (‘Qingnong dried No.2’) were obtained from the State Key Laboratory of Crop Genetics and Germplasm Enhancement in Qingdao Agricultural University. This cultivar was selected by researchers at Qingdao Agricultural University (Qingdao, China), the Qingdao Seed Station, and Dezhou Academy of Agricultural Sciences. Additionally, it was approved by the Shandong Variety Examination and Approval Committee in 2015 (deposition number: 2015–057-1). At first, the seeds were germinated in light incubator at 28°C. Three days later, the germinated seeds were transplanted into pot in a growth chamber with a photoperiod of 14 h of light and 10 h of darkness at 28°C/21°C. Six-leaf seedlings were used to treat with 100 μM Abscisic acid (ABA), 100 μM Methyl jasmonate (MeJA), 100 μM Salicylic acid (SA), 10% polyethylene glycol-6000 (PEG-6000), and 100 mM NaCl. High and low temperature were applied by placing seedlings in 38 and 4°C growth chamber, respectively. The leaf tissues were harvested at 0, 3, 6 and 12 h post various treatments. And all collected samples were immediately frozen in liquid nitrogen, and then stored at −80°C for RNA isolation. All samples were collected in triplicate from each of the sampling points. Besides, the samples of root, stem, leaf, flower, fruit and seed were harvested to investigate the tissue-specific expression.

**Total RNA isolation and cDNA synthesis**

Total RNA was isolated from plant materials using a total RNA kit (Tiangen, Beijing, China) according to the manufacturer’s instruction. A total of 1 μg of RNA of each sample was used for first-strand cDNA synthesis using M-MLV reverse transcriptase according to the manufacturer’s protocols (TaKaRa, Dalian, China). cDNA was diluted 20-fold for qRT-PCR analysis.

**Quantitative real-time PCR**

Primers were designed based on CaBBX gene sequences for real-time PCR by using the real-time PCR design tool in Integrated DNA Technologies (IDT, [https://www.idtdna.com/scitools/Applications/RealTimePCR/](https://www.idtdna.com/scitools/Applications/RealTimePCR/)) (All primers are listed in Table S1). Real-time PCR application was carried out in a LightCycler® 480 Real-Time PCR Detection System (Roche, Hercules, Switzerland) with ChamQ SYBR Color qPCR Master Mix (Vazyme, Nanjing, China). The constitutive actin gene (Gen Bank accession No. AY572427) was used as an internal control and served
as a standard gene for normalizing all mRNA expression levels [50]. A total of 20μL reaction system contained 10μL SYBR Color qPCR Master Mix, 1μL cDNA samples, 0.4μL of each primer (10μM) and 8.2μL ddH₂O. The PCR thermal cycle conditions were as follows: denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 10 s, and 58 °C for 20 s and 72 °C for 20 s. Fluorescence intensities were measured for qRT-PCR at the end of each cycle. A melting curve (61 cycles at 65 °C for 10 s) was performed directly to check for specific amplification. The relative gene expression was calculated by using the 2−ΔΔCT method [51], the experiments were performed triplicate technological repeats. The SPSS statistics software (version 17.0) was used to analyze significant differences [52].

Subcellular localization analysis
The subcellular localization of CaBBX proteins was predicted by Plant-mPLoc in Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) [53]. And four CaBBX proteins were chosen to verify the prediction results. The four selected CaBBX genes were isolated from the cDNAs of pepper var. ‘Qingnong dried tomato’ and the amplified products were recombined into pMDC83 vector with green fluorescent protein (GFP), and then transferred to Agrobacterium tumefaciens Gv3101 strain for following infection. The onion inner epidermis was used for transforming with vectors with CaBBX genes. Fluorescence images were captured and analyzed using a Zeiss laser scanning confocal microscope TCS SP5 (Leica, Brunswick, Germany) and the LSM image software.

Results
Identification and characteristics of BBX genes in pepper
We searched PepperHub (Pepper Information Hub, http://pepperhub.hzau.edu.cn/) and PGP (Pepper Genome Platform, http://passport.pepper.snu.ac.kr/?t=PGENOME) with the conserved B-box domain HMM profile (PF00643) to obtain global putative BBX genes in pepper. Then the putative encoding protein sequences of these genes were further confirmed their B-box domain by using SMART, Pfam and InterProScan searches, six putative genes without B-box domain were removed. In total, we eventually identified 24 BBX genes in pepper, which were named CaBBX1 to CaBBX24. Afterwards, the detailed information gene name, gene annotation ID, genomic position, gene length, theoretical isoelectric point, and molecular weight of their encoding protein were listed in Table 1.

These BBX genes showed diverse in length leads to the various length, theoretical isoelectric point, and molecular weight of their encoding protein. These BBX genes with sequence of 582 to 1476bp encoded ranging from 193 (least, CaBBX7) to 491 (most, CaBBX22) amino acid residues. And the isoelectric points of 24 BBX proteins were ranging from 4.60 (lowest, CaBBX23) to 9.17 (highest, CaBBX24), with the molecular weights of 21.20 ~ 54.98kDa (Table 1).

Phylogenetic analysis of the CaBBX family
To identify the phylogenetic relationship and division of CaBBX proteins, we constructed the phylogenetic tree of BBX family proteins in pepper (Fig. 1). The phylogenetic analysis of the CaBBXs with AtBBXs, PtBBXs, OsBBXs and SIBBXs was also carried out to confirm the subfamily clustering of CaBBXs (Fig. S1). The division of 24 CaBBXs were not even on the phylogenetic tree (Fig. 1A). All the 24 CaBBXs were divided into five subfamilies with the similarity of the amino acid sequences based on previous studies in tomato [1]. In addition, the phylogenetic relationship of first B-box domain was constructed, as well as two B-box and one CCT domain (Fig. 1B and C). In total, there were eight CaBBXs classified into subclass I, whose contain two B-box domains, making up the largest subclass. The subclass II and III both contained six members, and only two members (CaBBX12 and 13) clustered together in subclass IV, and CaBBX23 and 24 aligned together in subclass V. Members from subclass II owed two B-box domains and one CCT domain, while only one BBX proteins (CaBBX13) possessed one B-box and one CCT domain belonging to subclass IV. Other CaBBX proteins only contained one or two B-box domains without CCT domain. Moreover, based on the phylogeny of BBXs in Arabidopsis, rice, tomato and Populus tomentosa, most of the BBXs with two B-box domains and one CCT domain were classified into subgroup II, and most of whom with two B-box domains and none CCT domain were classified into subgroup I. While, BBXs contain one single B-box domain were most together classified into subgroup V.

Domains, motif structure and gene structure analysis
To determine the domains, motif structure and gene structure of CaBBXs, the conserved domain information were confirmed by CDD in NCBI, and motif and CDS were also plotted to identify structure analysis of CaBBXs (Fig. 2).

Eight motifs were identified in these CaBBXs, members from same subclass shared similar motifs according to the phylogenetic relationship (Fig. 2A). For example, all the members from subclass I contained motif 1, 3 and 7, only CaBBX20 also owed another motif 6. While, except CaBBX4 only owed two motif 1 and one motif 2, CaBBXs from subclass II possessed maximum motifs, containing motif 1, 2, 3, 5 and 7; moreover, three of them...
(CaBBX1, 2 and 3) contained all the 8 identified motifs. Other members carried one or two motif 1, several of them contained motif 2; in addition, CaBBX11 and 13 also had motif 4 and 8 located in middle of their amino acid sequence, respectively. The detail sequence information of these eight motifs were shown in Fig. S2.

Furthermore, the gene structures of CaBBXs were constructed with TBtools by gff file from pepper genome 2.0 [49]. Among 24 CaBBXs, only CaBBX24 had no intron, others had one to five exons. To make clear the domains arrangement, we also plotted domains on the CDS directly. Nine BBX proteins were identified containing two B-box and a CCT domain, five of them share two same B-box, and four possessed two different B-box domains. Only one BBX proteins (CaBBX13) possessed one B-box and one CCT domain, while three and eleven CaBBX proteins contained one and two B-box without CCT domain, respectively (Fig. 2B). These results were consistent with the phylogenetic divergence analysis. Except for subclass I members, the B-box domains of members from other subclasses were located in the beginning of first exon. The B-box domains of subclass I members were on the first three exon. CCT domain were situated in the terminal of last two exon. Moreover, the two B-box (B-box1 and B-box2 domains) share similar conserved sequences and Zinc finger domain.

Chromosomal localization and duplication of BBX genes in pepper

We have plotted the CaBBX genes to the chromosomes of pepper genome to confirm their genomic distribution (Fig. 3). Except for four CaBBX genes (CaBBX7, CaBBX9, CaBBX11 and CaBBX24), 20 CaBBX genes were distributed unevenly on 11 of 12 pepper chromosomes, no gene was on chromosome 10. Both chromosome 02 and 07 possessed four CaBBX genes, making up the maximum number of genes among all these 12 chromosomes. In addition, only one CaBBX gene was located on chromosome 01, 04, 05, 06, 09 and 11, respectively; and two on chromosome 03, 08 and 12, respectively.

Potential duplication within pepper were marked on the 12 chromosomes by using TBtools [49]. Expect CaBBX7 (duplicated with CaBBX8) was not located on pepper chromosomes, the other three duplications

Table 1 Information of the BBX gene family in pepper

| Gene   | Annotated CDS     | Genomic position       | Chr | CDS   | AA   | pIs   | MV    | Subcellular localization |
|--------|-------------------|------------------------|-----|-------|------|-------|-------|--------------------------|
| CaBBX1 | Capana02g003201   | 157,124,787–157,126,407 | 2   | 1224  | 407  | 5.27  | 43.89 | Nucleus                  |
| CaBBX2 | Capana02g003200   | 157,118,313–157,120,062 | 2   | 1197  | 398  | 5.32  | 45.39 | Nucleus                  |
| CaBBX3 | Capana02g003199   | 157,107,846–157,110,095 | 2   | 1215  | 404  | 5.41  | 44.56 | Nucleus                  |
| CaBBX4 | Capana01g004030   | 278,243,944–278,245,872 | 1   | 1032  | 343  | 5.45  | 37.93 | Nucleus                  |
| CaBBX5 | Capana12g000414   | 8,179,392–8,181,909     | 12  | 1071  | 356  | 5.29  | 39.43 | Nucleus                  |
| CaBBX6 | Capana07g000300   | 1,563,552–1,565,426     | 7   | 1155  | 384  | 6.56  | 42.54 | Nucleus                  |
| CaBBX7 | Capana00g004028   | 606,409,675–606,410,256 | –   | 582   | 193  | 5.32  | 21.20 | Nucleus                  |
| CaBBX8 | Capana07g001114   | 154,056,816–154,057,654 | 7   | 693   | 230  | 5.03  | 24.83 | Nucleus                  |
| CaBBX9 | Capana00g004489   | 649,683,777–649,686,080 | –   | 1281  | 426  | 5.07  | 46.87 | Nucleus                  |
| CaBBX10| Capana03g003558   | 228,734,754–228,738,294 | 3   | 1164  | 387  | 5.45  | 43.34 | Nucleus                  |
| CaBBX11| Capana00g001486   | 399,610,115–399,614,902 | –   | 1410  | 469  | 6.75  | 51.67 | Nucleus                  |
| CaBBX12| Capana11g002294   | 218,148,220–218,150,401 | 11  | 951   | 316  | 4.85  | 35.20 | Nucleus                  |
| CaBBX13| Capana03g000577   | 5,273,465–5,275,489     | 3   | 1149  | 382  | 5.53  | 43.46 | Nucleus                  |
| CaBBX14| Capana02g002620   | 148,180,402–148,183,481 | 2   | 624   | 207  | 8.47  | 22.97 | Nucleus                  |
| CaBBX15| Capana08g002625   | 149,926,925–149,929,248 | 8   | 639   | 212  | 6.17  | 23.52 | Nucleus                  |
| CaBBX16| Capana12g006559   | 18,213,299–18,215,385   | 12  | 960   | 319  | 6.50  | 35.22 | Nucleus                  |
| CaBBX17| Capana04g002666   | 4,091,269–4,092,722      | 4   | 918   | 305  | 6.24  | 33.86 | Nucleus                  |
| CaBBX18| Capana07g002662   | 212,563,126–212,570,473 | 7   | 894   | 297  | 4.98  | 32.01 | Nucleus                  |
| CaBBX19| Capana09g005394   | 12,877,150–12,887,608   | 9   | 900   | 299  | 4.97  | 32.17 | Nucleus                  |
| CaBBX20| Capana09g000735   | 11,697,317–11,699,646    | 6   | 702   | 233  | 5.00  | 25.98 | Nucleus                  |
| CaBBX21| Capana08g002611   | 149,745,414–149,746,937 | 8   | 804   | 267  | 5.62  | 29.83 | Nucleus                  |
| CaBBX22| Capana05g001195   | 84,622,805–84,628,926    | 5   | 1476  | 491  | 6.00  | 54.98 | Nucleus                  |
| CaBBX23| Capana07g001588   | 191,773,542–191,774,577 | 7   | 744   | 247  | 4.60  | 27.40 | Nucleus                  |
| CaBBX24| Capana00g004911   | 672,862,631–672,863,374 | –   | 744   | 247  | 9.17  | 27.71 | Nucleus                  |

Note: Annotated CDS annotated coding DNA sequences, Genomic position, Chr chromosome, CDS coding DNA sequences, AA amino acid residues, pI theoretical isoelectric point, MV, Subcellular localization. The subcellular location results of pepper BBX genes were predicted by Plant-mPLoc in Cell-PLoc 2.0.
only occurred on the 3 of 12 chromosomes, and these duplicated genes (CaBBX14, CaBBX15, CaBBX17 and CaBBX21) were all belonged to subgroup I (Fig. 3). And all the duplication events occurred between two different chromosomes, not within the same chromosome. In addition, we constructed a collinearity relationship analysis to identify the duplication events of BBX genes between pepper and the model Solanaceae plant tomato (Fig. 4). Twenty-six pairs of BBX genes were identified duplicate between pepper genome and tomato genome. All the subgroups of BBX genes involved in duplication. Among them, 13 pairs of subgroup I members play part in the replication events, account for half of the total duplication events. And we found 4 pairs of subgroup II and III members, 3 pairs of subgroup IV members and 2 pairs of subgroup V members were homologous in the pepper and tomato genome (Fig. 4).

Organ development expression analysis of BBX genes in pepper

To investigate the tissue-specific and developmental expression pattern of all the CaBBX genes, we performed the heatmap by using TBtools based on the transcript data from Pepper Information Hub (http://pepperhub.hzau.edu.cn/) [49]. Several CaBBX genes showed organ-specific expression pattern, such as CaBBX19, expressed specifically in seed, respectively, and expressed arise as the tissues’ development (Fig. 5). This result indicated that CaBBX19 may play an important role in seed morphogenesis development, respectively. CaBBX7, CaBBX12, CaBBX13 and CaBBX22 mainly expressed in leaf, may showed their regulatory function in pepper leaf (Fig. 5). Additionally, CaBBX5 and CaBBX6 showed high expression levels in leaf and flower, CaBBX3 and CaBBX14 specifically expressed in the early developmental stage.
of flower, CaBBX4 and CaBBX20 expressed in almost all the detected tissues, and expressed most highly in fruit development, especially in the pericarp, however, expect in the seed (Fig. 4). This result may indicate that CaBBX4 and CaBBX20 involved in the pericarp development (such as pigmentation, enlargement, and so on).

Furthermore, we also investigated the expression levels of 24 CaBBX genes by qRT-PCR. In particularly,
CaBBX19 showed the highest expression level in seed, and CaBBX24 was expressed more highly in flower
Fig. 6  The expression of CaBBXs in different pepper tissues. Different tissues were arranged as 'root, stem, leaf, flower, fruit and seed'. Three independent biological experiments were performed ($P < 0.05$)
Expression analysis of BBX genes under abiotic stress in pepper

The expression levels of CaBBX genes under cold, heat, salt and drought stress were investigated by qRT-PCR analysis, to analyze CaBBX genes in response to abiotic stress (Fig. 7). We selected six CaBBX genes (CaBBX3, CaBBX4, CaBBX5, CaBBX6, CaBBX13 and CaBBX20) potentially responding to abiotic stress base on transcriptome data of different stress treatment from the Pepper Genome Database (Fig. S3, http://pepperhub.hzau.edu.cn/). The expression levels were detected under high temperature, low temperature, NaCl and PEG6000 treatment at 3h, 6h, 12h. In low temperature treatment, four of these six CaBBX genes showed up-regulated expression, except for CaBBX13 and CaBBX20, they both showed a high expression level under 3h cold treatment, but rapidly down-regulated afterwards 6h (Fig. 7A). Under heat stress condition, the expression of CaBBX4, CaBBX5, CaBBX6 and CaBBX13 got a peak at 3 or 6h treatment, and then decreased at 12h treatment, while CaBBX3 and CaBBX20 exhibited the opposite expression pattern (Fig. 7B). There were three CaBBX genes (CaBBX4, CaBBX5 and CaBBX6) expressed up-regulated under drought stress, and the expression levels of other three genes were decreased (Fig. 7C). Only the expression of CaBBX3 was repressed under salt condition, and showed an early down-regulation at 3 and 6h, but rapidly increased its expression even more so than the control at 12h, while CaBBX4, CaBBX5, CaBBX6 and CaBBX20 were expressed more highly than control. Additionally, CaBBX13 showed an up-regulation at 6 h, but rapidly decreased with a more lower expression level than control at 12 h treatment (Fig. 7D). These results indicated that the six CaBBX genes may involve in responding to abiotic stress.

Expression analysis of CaBBX genes in response to exogenous hormones

In addition, the expression pattern of CaBBX genes under ABA, MeJA and SA treatment were also measured because of their important part in plant growth, development and in response to biotic and abiotic stress. The expression profiles of six CaBBX genes potentially involved in response to abiotic stress were also investigated under ABA, MeJA and SA treatment at 3h, 6h, 12h (Fig. 8). Three of these detected genes (CaBBX4, CaBBX13 and CaBBX20) were induced to express more highly by ABA treatment than control at different time-point treatment. CaBBX4 was up-regulated during the entire treatment. However, the expression level of CaBBX13 was increased significantly at 3 and 6h treatment, but was repressed at 12h treatment. And the expression of CaBBX3 was repressed by ABA significantly at all the three time-point (3, 6 and 12h) treatment. CaBBX5 showed no obviously significant expression levels, and the expression of CaBBX6 at 3 and 12h treatment was lower than that of control (Fig. 8A). The expression of CaBBX3, CaBBX4 and CaBBX20 were repressed dramatically after MeJA application, while the expression of other three genes (CaBBX5, CaBBX6 and CaBBX13) were up-regulated at early (3h and/or 6h) treatment stage, but CaBBX6 and CaBBX13 were expressed decreased rapidly at 12h treatment (Fig. 8B). Except for CaBBX3 and CaBBX13, all the other four selected CaBBX genes were induced to expressed at a higher level by exogenous SA, and reached peak at 6h treatment. Except for CaBBX3 and CaBBX5, all the other four selected CaBBX genes were induced to expressed at a higher level by exogenous SA at different treatment stages (Fig. 8C).

Subcellular localization of CaBBXs

The subcellular localization of proteins was analyzed to further understand the function. We first predicted the subcellular localization by Plant-mPLoc in Cell-PLoc 2.0 (Table 1). All of the 24 CaBBXs were identified to be located in nucleus with the highest possibility. We selected three CaBBXs (CaBBX5, 6, and 20) which were strongly induced or repressed by abiotic stress, hormones or showed organ-specific and organ developmental expression patterns for a transient expression assay using GFP-fused BBX proteins with onion epidermis. All the three CaBBXs were found to be in the nucleus (Fig. 9). The result of CaBBX20, CaBBX5 and 6 was consistent with the most preferentially predication.

Discussion

Genome evolution of the B-box transcription factors in pepper

BBX transcription factors were widely identified in many higher plants, such as Arabidopsis [2], rice [11], tomato [10], apple [13], pear [12], and so on [14, 42, 54]. The CaBBX was characterized the structure, phylogenetic relationship, chromosomal location, expression patterns and subcellular localization in our study. Based on the sequence similarity, the BBXs can be classified into five different subclasses [25]. The BBX genes were investigated in 13 different high plants, and the total number among these plant species is relative stable with about 30 members (Fig. S4), expect for several...
Fig. 7 The expression of CaBBXs under different abiotic stresses. 

A. qRT-PCR transcript analysis of 6 selected CaBBX genes under cold stress.

B. qRT-PCR transcript analysis of 6 selected CaBBX genes under heat stress.

C. qRT-PCR transcript analysis of 6 selected CaBBX genes under drought stress.

D. qRT-PCR transcript analysis of 6 selected CaBBX genes under salt stress. Three independent biological experiments were performed (P < 0.05)
Fig. 8 The expression of CaBBXs under different hormone treatment. A. qRT-PCR transcript analysis of 6 selected CaBBX genes under ABA treatment. B. qRT-PCR transcript analysis of 6 selected CaBBX genes under MeJA treatment. C. qRT-PCR transcript analysis of 6 selected CaBBX genes under SA treatment. Three independent biological experiments were performed (P < 0.05).
species which experienced whole genome duplication. The triplication events occurred in Chinese cabbage genome since its divergence from Arabidopsis, resulting in the genome size of Chinese cabbage (485 Mb) more than three times larger than that of Arabidopsis (125 Mb) [55]. However, there were 75 BBX members in Chinese cabbage, only twice of that in Arabidopsis (32 members). Moreover, the number of GmBBXs (50 members) is more than three times of Medicago truncatula (15 members), soybean has undergone a specific tetraploidy [56]. The gene number is less than the genome duplication ploidy may result from alternative actions during the evolution. The number of BBX genes in the three Solanaceae species (tomato, pepper, and potato) is relatively stable. However, the number of each subfamily among these plants differed a lot, this may indicate that the function of BBX belonging to different subclasses varied resulting from the differentiation of B-box proteins.

In addition, the diversity of are motif and intron-exon structure important for the evolution of many gene families. In subclass I, except for CaBBX20, all the other six CaBBXs had six same motifs (motif 1, 3 and 7). And CaBBX1–3 in a clustering clade within subclass II, contained all the same motif structure (Fig. 2A). It was identified that the CaBBXs in same phylogenetic clade shared the similar motif structures resulting in a conserved pattern in the evolution of CaBBX transcription factors.

Moreover, we found that the intron-exon structure of CaBBXs in same subclass also differed a little between the diverse clades, but are highly similar within the same phylogenetic clade. Thus, it is also indicated that the evolution of CaBBXs were relatively conserved with low occurrence of mutation events. However, the full genomic sequences of most CaBBX genes were below 5 kb, but not for that of CaBBX18, 19 and 22 that were 6 kb, 7 kb and 10 kb, respectively (Fig. 2B). Therefore, mutation events might still exist in highly conserved genes resulting from the evolutionary diversity.

Gene duplication of BBX genes in pepper
As is known, genetic novelty mainly caused by gene or genome duplication events, and gene family expansion was primarily resulting from gene duplication [57–59]. Novel BBX genes arise through divergence of duplicate genes after either single gene duplication, segmental duplication, or whole-genome duplication [57, 60]. The chromosomal location of CaBBX genes indicated...
that the distribution of BBX genes in pepper genome is not even, this result may cause by genome duplication which occurred throughout plant evolution [61]. Three pairs of potential duplicate BBX genes were found in pepper chromosomes, and a single CaBBX gene, CaBBX7, was located on ChrUM. Interestingly, all the three pairs of duplicate genes belonged to the same subgroup I, and CaBBX14 duplicated with CaBBX15 and CaBBX17. While CaBBX17 duplicated with CaBBX21, which was clustered with CaBBX15 located on chromosome 08 (Fig. 3). In addition, 26 CaBBX-SlBBX gene pairs were identified by collinearity analysis. Among them, 13 subgroup I gene pairs were found counted for the largest number of replication events. These results suggest that the expansion of CaBBX genes in pepper chromosome is affected by the network and duplication events of pepper.

The evolution of CaBBX transcription factors might indicate that their diverse function in tolerance to abiotic and biotic stress, responding to phytohormone, and even in plant growth and developmental processes, such as seedling photomorphogenesis, shade avoidance, photoperiodic regulation of flowering [25]. And the specific function of CaBBX transcription factors involved in plant development and stress tolerance were still looking forward to be elucidated.

**Tissue-specific and developmental expression patterns of CaBBX genes**

In several model plant, Arabidopsis, rice or tomato, the BBXs participated in seedling photomorphogenesis, such as flowering, hypocotyl growth, pigmentation and cotyledon unfolding [8, 27, 33, 62, 63]. Here, the transcript expression levels of 24 CaBBXs were investigated in ten organs or tissues, as well as during leaf, flower, pulp, placenta and seed development (Fig. 5). Over-expression of CONSTANS-LIKE 5 can induce flowering under short-day condition in Arabidopsis [28]. AtBBX30 and AtBBX31 were negatively regulated by HY5, which directly binding to the G-box cis-element present in their promoters, negatively regulate photomorphogenesis in Arabidopsis [22]. While the homology of AtBBX30 and AtBBX31, CaBBX24 was expressed at a high level in flower (Figs. 5 and 6). AtBBX4 is a key component involved in the phyB (Phytochrome B)-PIF3 (PHYTOCHROME INTERACTING FACTOR 3) regulatory module to promote photomorphogenesis [23]. CaBBX4 clustered with AtBBX4, expressed highly in flower and pulp, while the flower and fruit development were related to photomorphogenesis. OsCO3, a BBX gene in rice, can regulating flowering time by repressing the expression of FT-like genes under SD conditions [30]. Recent study suggested that AtBBX28 negatively regulates photomorphogenesis by repressing HY5 activity [21], the homologous BBX in pepper is CaBBX23, showed lower expression in flower in RNA-seq data transcript levels and qRT-PCR analysis (Figs. 5 and 6), with the similar function of AtBBX28. CaBBX7, 12 and 13 were expressed a relatively high level in the early stage of leaf development, may suggest they involved in cotyledon unfolding. In addition, CaBBX5 and 6 were also expressed at a high level in both leaf and flower, suggesting they were involved in photomorphogenesis. CaBBX19 expressed only in seed, and had a gradually increasing expression pattern during seed development, indicating that its important roles in seed formation and growth.

Besides, AtBBX24 and AtBBX25 were identified interacting with HYH, an HY5 HOMOLOG, to regulate Arabidopsis seedling development [64], AtBBX24 and AtBBX25 were clustering with CaBBX20 by our phylogenetic analysis (Fig. S1). Recently, a tomato BBX transcription factor, SIBBX20 modulates fruit pigmentation by directly activating the rate-limited enzyme of carotenoid biosynthesis PSY1 [62]. CaBBX20 was classified into group I homologous with AtBBX24 and AtBBX25, and expressed a relatively high level in pepper pulp (Fig. 4), this may indicate that CaBBX20 evolved special function in fruit development.

**Stress and hormones induced expression of BBX genes in pepper**

BBX transcription factors were also proved to be involved in response to stress and phytohormones [27, 35]. AtBBX18 was detected to be a negative regulator in heat tolerance in Arabidopsis [39]. In pepper, we found the expression profiles of CaBBX4, 5, 6, 13 and 20 were significantly similar to be induced high expression by heat and salt stress. Moreover, CaBBX20 homologous with AtBBX24, was induced by salt stress (Fig. 7D), AtBBX24 also called STO, was identified to be a salt tolerance factor, which can enhance Arabidopsis root growth under salt stress treatment [35]. And it was found that SIBBX20 was up-regulated in M82 (cultivated tomato M82 is sensitive to stress) under drought stress in tomato [65]. Similarly, CaBBX4 was dramatically up-regulated (up to 10~60 fold comparing with control) under drought stress, may indicated that they shared the similar function in responding to drought stress.

Phytohormones are important for plant growth, development and also involved in tolerance to biotic and abiotic stress. Recently, an apple B-box protein BBX37 was identified that regulates jasmonic acid mediated cold tolerance through the JAZ-BBX37-ICE1-CBF pathway [41], MdBBX10 significantly enhanced abiotic stresses tolerance by ABA signalling [66].BBX19 belonged to subgroup IV, interacts with ABF3 to affect drought tolerance negatively in chrysanthemum [67]. All these selected BBX genes were found to be responded to ABA, MeJA and
SA. Except for CaBBX3, other five CaBBX genes were up-regulated expressed by ABA and SA application. This result may indicate that CaBBX4, 5, 6, 13 and 20 as positive factors response to ABA and SA signaling involved in pepper plant growth or biotic and abiotic stress tolerance. While, under MeJA condition, only CaBBX5 showed up-regulated expression at all three treated stages, others were down-regulated at different degrees, especially CaBBX3 and 4. This result may indicate that CaBBX3, 4, 6, 13 and 20 as negative factors response to later stage of MeJA signaling.

Conclusion

In this study, we carried out a genome-wide analysis of 24 CaBBX genes, the phylogenetic analysis, domain, motif & gene structure, gene chromosome location, were performed preliminarily. In addition, several CaBBX genes were induced by abiotic stress and exogenous phytohormones, some expressed tissue-specific and variously at different developmental stage. And subcellular localization experiment was also investigated to further understand the potential function of CaBBX genes, it was indicated that they act as nucleus-localized transcription factors. Overall, our data might be a foundation in the identification of CaBBX genes, and a further understanding of their biological function in future studies.

Supplementary Information

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Authors' contributions

JM, DL, JXD and XWL conceived, designed and wrote the manuscript. JM analyzed data. JXD performed all experiments. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article (and its supplementary information files) or are available from the corresponding author on reasonable request. Sequence data from this article can be found in Arabidopsis Information Resource (https://www.arabidopsis.org/) under the following accession numbers: ABX81 (AT3G15840), ABX82 (AT3G15850), ABX83 (AT3G02380), ABX84 (AT2G24790), ABX85 (AT3G24930), ABX86 (ATSG57660), ABX87 (AT3G07650), ABX88 (ATSG48250), ABX89 (AT4G15250), ABX90 (AT3G21880), ABX91 (AT2G34780), ABX92 (AT3G33500), ABX93 (AT2G80500), ABX94 (AT1G25000), ABX95 (AT1G5440), ABX96 (AT1G37870), ABX97 (AT1G49130), ABX98 (AT2G21320), ABX99 (AT4G38960), ABX100 (AT4G39070), ABX101 (AT1G57440), ABX102 (AT7G8600), ABX103 (AT1G0240), ABX104 (AT1G6040), ABX105 (AT2G31380), ABX106 (AT1G62550), ABX107 (AT1G68190), ABX108 (AT2G37310), ABX109 (AT4G54730), ABX110 (AT4G15248), ABX111 (AT3G21890), ABX112 (AT9G21150). The sequences of vVBBX1 (VT_214s0083g00640.2), vVBBX2 (VT_211s0052g01000.1), vVBBX3 (VT_204s0008g07340.1), vVBBX4 (VT_212s0057g01350.2), vVBBX5 (VT_200s0194g00701.1), vVBBX6 (VT_207s0104g01360.1), vVBBX7 (VT_201s0146g03601.1), vVBBX8 (VT_214s0068g01380.1), vVBBX9 (VT_201s0110g02401.1), vVBBX10 (VT_219s0014g05120.1), vVBBX11 (VT_212s0059g02500.1), vVBBX12 (VT_201s0011g03520.1), vVBBX13 (VT_203s0038g00690.1), vVBBX14 (VT_204s0023g00300.1), vVBBX15 (VT_218s0001g13520.1), vVBBX16 (VT_202s0039g00140.1), vVBBX17 (VT_216s0089g01280.1), vVBBX18 (VT_201s0044g03015.1), vVBBX19 (VT_205s0102g07970.1), vVBBX20 (VT_201s0144g03060.1), vVBBX21 (VT_212s0134g00400.1), vVBBX22 (VT_212s0059g02510.1), vVBBX23 (VT_205s0203g00210.1), vVBBX24 (VT_209s0054g00530.1) are available in Genome Annotation Batch Download of Grape Genome Annotation Project (http://genomes.cribi.unipd.it/grape/). The sequences of ZmBBX1 (zma100147736), ZmBBX2 (zma100281837), ZmBBX3 (zma100282189), ZmBBX4 (zma100281883), ZmBBX5 (zma100383382), ZmBBX6 (zma100281064), ZmBBX7 (zma100281114), ZmBBX8 (zma100193195), ZmBBX9 (zma100304421), ZmBBX10 (zma100282916), ZmBBX11 (zma100383648), ZmBBX12 (zma100283083), ZmBBX13 (zma100272654), ZmBBX14 (zma100263174), ZmBBX15 (zma100383388), ZmBBX16 (zma10027363), ZmBBX17 (zma1002283103), ZmBBX18 (zma100294784), ZmBBX19 (zma100273973), ZmBBX20 (zma100274169), ZmBBX21 (zma100193074), ZmBBX22 (zma100284380), ZmBBX23 (zma100285359) are available in Genome Annotation Batch Download of maize Genome Annotation Project (http://www.maizesequence.org/index.html). The sequences of PpBBX1 (PPSTR_66207), PpBBX2 (PPSTR_831202), PpBBX3 (PPSTR_834782), PpBBX4 (PPSTR_737847), PpBBX5 (PPSTR_562026), PpBBX6 (PPSTR_737488), PpBBX7 (PPSTR_836808), PpBBX8 (PPSTR_658939), PpBBX9 (PPSTR_419707), PpBBX10 (PPSTR_752788), PpBBX11 (PPSTR_569981), PpBBX12 (PPSTR_852783), PpBBX13 (PPSTR_846848), PpBBX14 (PPSTR_243653), PpBBX15 (PPSTR_774835), PpBBX16 (PPSTR_851021), PpBBX17 (PPSTR_247140), PpBBX18 (PPSTR_564570), PpBBX19 (PPSTR_868551), PpBBX20 (PPSTR_782168), PpBBX21 (PPSTR_747119), PpBBX22 (PPSTR_721866), PpBBX24 (PPSTR_648693), PpBBX25 (PPSTR_550941), PpBBX26 (PPSTR_801330), PpBBX27 (PPSTR_804174), PpBBX28 (PPSTR_855496), PpBBX29 (PPSTR_1094062), PpBBX30...
The sequences of SIBBX1 (Solyc02g089520.1), SIBBX2 (Solyc12g089240.1), SIBBX21 (Solyc04g081020.2), SIBBX22 (Solyc07g062160.2), SIBBX23 (Solyc12g005420.1), SIBBX24 (Solyc06g073180.2), SIBBX25 (Solyc01g110180.2), SIBBX26 (Solyc10g066750.2), SIBBX27 (Solyc04g074930.2), SIBBX28 (Solyc12g005660.1), SIBBX29 (Solyc12g079430.2) are available in locus search of Sol Genomics Network (SGN: https://www.sgn.cornell.edu) database, and CaBBX1 (Capano02g003201), CaBBX2 (Capano02g003200), CaBBX3 (Capano02g003199), CaBBX4 (Capano01g004030), CaBBX5 (Capano12g000144), CaBBX6 (Capano07g000130), CaBBX7 (Capano07g000428), CaBBX8 (Capano07g001114), CaBBX9 (Capano07g000489), CaBBX10 (Capano03g003558), CaBBX11 (Capano10g001486), CaBBX12 (Capano11g002794), CaBBX13 (Capano03g000377), CaBBX14 (Capano02g002620), CaBBX15 (Capano08g002625), CaBBX16 (Capano12g000569), CaBBX17 (Capano04g000266), CaBBX18 (Capano17g002062), CaBBX19 (Capano09g000394), CaBBX20 (Capano06g000730), CaBBX21 (Capano08g000611), CaBBX22 (Capano05g001195), CaBBX23 (Capano07g001588), CaBBX24 (Capano04g000491) can be downloaded from the Pepper Genome Platform (PGP: http://peppergenome.snu.ac.kr/download.php).

Declarations

Ethics approval and consent to participate
Not applicable. This study was conducted in accordance with the People’s Republic of China and international authorities’ relevant guidelines and legislation, including the official website of the Committee on Publication Ethics (http://www.publicationethics.org/) and the European Association of Science Editors (EASE) and other institutions’ publishing ethics standards. Pepper seeds (“Qingnong dried No.2”) were obtained from the State Key Laboratory of Crop Genetics and Germplasm Enhancement in Qingdao Agricultural University. This cultivar was selected by researchers at Qingdao Agricultural University. Following the approval by the Shandong Variety Examination and Approval Committee in 2015 (deposition number: 2015–057–1).

Consent for publication
Not applicable.

Competing interests
The authors declare that there are no competing interests.

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