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Genome-Wide Survey and Expression Analysis of B-Box Family Genes in Cucumber Reveal Their Potential Roles in Response to Diverse Abiotic and Biotic Stresses

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Abstract: As a class of zinc finger transcription factors, B-box (BBX) proteins play diverse roles in numerous biological processes, and they have been identified in a series of plant species in recent years. However, the roles of *BBX* genes in regulating cucumber growth regulation and stress response have not yet been established. Here, a total of 22 *BBX* family genes were identified via an analysis of the latest cucumber genome data, which were classified into five groups (I–V) on the basis of their phylogenetic features and number of B-box domains and CCT domains. The *CsBBX* genes were unevenly distributed across the seven cucumber chromosomes, and segmental duplication was found to play a significant role in the expansion of the cucumber *BBX* gene family. Gene structure and motif composition analysis suggested that the evolutionarily close CsBBXs have similar conserved motif composition and gene structure. Most *CsBBX* genes possessed 1–3 introns, and intron gain rather than intron loss could contribute to the different structures of *CsBBX* genes across different groups during their evolution. Promoter analysis revealed the presence of 13 kinds of hormone-related and nine kinds of stress-related *cis*-regulatory elements in the promoter regions of these *CsBBX* genes. Expression analysis via RNA-seq and qRT-PCR suggested that the *CsBBX* genes exhibit differential expression in different tissues and in response to various abiotic and biotic stresses. This work constitutes a starting point for further revealing the function of the *CsBBX* genes and sheds light on the potential molecular mechanism of stress resistance in cucumber.

Keywords: cucumber; B-box (BBX); stress; gene expression profiling; gene duplication

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1. Introduction

Zinc finger transcription factors (TFs), which are widely present in all eukaryotes, are vital regulators playing important regulatory roles in various essential biological processes of plants. B-box (BBX) proteins are a class of zinc finger TFs characterized by the presence of one or two specific B-box domains with approximately 40 amino-acid residues at the N-terminus [1,2]. On the basis of the consensus sequence and specificity of the zinc ion binding site, the B-box domains can be divided into two types, namely, B-box 1 and B-box 2 [2,3], both of which are highly conserved across species and have certain functions in the protein–protein interaction between BBXs and other proteins, such as TOPLESS [4], early flowering 3 (ELF3) [5], ABF [6], and LONG HYPOCOTYL 5 (HY5) [7–9]. In addition, some BBX proteins contain a conserved CCT (CONSTANS, CO-like, and TOC1) domain at the C-terminus, which is involved in nuclear protein transport, transcriptional regulation, and protein–protein interaction. For instance, the CCT domains of CO and HEADING

DATE1 (HD1) can form a heterotrimer with NF-YB/YC (NUCLEAR FACTOR-YB/YC) dimers, and then specifically bind to a conserved 'CCACA' motif upstream of the promoter regions of their target genes [10,11]. Members of the BBX family in various plant species can be classified into five groups (I–V) on the basis of their numbers of B-box and CCT domains, with group I and group II having one CCT domain and two B-box domains, group III comprising one CCT domain and one B-box domain, group IV including two B-box domains, and group V having one B-box domain [2,12].

BBX proteins are known to participate in a wide range of physiological processes in plants. For example, four members of group IV in *Arabidopsis* (BBX18, BBX19, BBX24, and BBX25) negatively regulate photomorphogenesis, while three other members in this group (BBX20, BBX21, and BBX22) positively regulate photomorphogenesis [13,14]. In addition, some BBXs are associated with light-induced proanthocyanidin biosynthesis and anthocyanin accumulation. For example, PpBBX16 and PpBBX18 can positively regulate anthocyanin synthesis by interacting with PpHY5, while PpBBX21 interferes with the formation of the PpBBX18–PpHY5 heterodimer, thereby repressing anthocyanin biosynthesis [15,16]. In apple, MdBBX21 can also interact with MdHY5 and observably enhance the promoter activity of *MdMYB1* under light, thereby regulating anthocyanin accumulation [17]. Under light stress, MdBBX22 can bind to the promoter of mdm-miR858, inducing its transcription to inhibit proanthocyanidin accumulation and subsequently indirectly promoting anthocyanin synthesis in the peel [18]. Furthermore, UV-B can promote the transcription of MdBBX22 via both transcriptional and post-translational regulations, thereby acting as a cofactor of MdHY5 to activate UV-B-induced anthocyanin biosynthesis [19]. Moreover, numerous reports have documented that BBXs also play crucial roles in diverse developmental processes, such as seed germination [20], shade avoidance [21], leaf senescence [22,23], and circadian regulation [24], as well as in the responses of plants to various biotic and abiotic stresses. For example, BBX18 and BBX23 can repress the protein levels of ELF3 and, therefore, positively regulate thermomorphogenesis in *Arabidopsis* [5]. Additionally, BBX18 promotes thermomorphogenesis by interfering with PSEUDO-RESPONSE REGULATOR 5 (PRR5)-mediated inhibition of PIF4 in response to high temperature [25]. A tomato BBX member SlBBX17 acts as a negative regulator of plant growth but positively regulates heat tolerance [26]. Overexpression of a chrysanthemum *BBX* gene *CmBBX22* in *Arabidopsis* delayed leaf senescence and improved drought tolerance [22]. Similarly, transgenic *Arabidopsis* plants overexpressing apple *MdBBX1* and *MdBBX10* exhibited enhanced tolerance to multiple abiotic stresses [27,28].

Cucumber, a popular and economically important crop of the Cucurbitaceae family, is widely cultivated in many countries. It produces tender fruits rich in nutrients and provides people with various health-promoting properties. However, its growth and development are always negatively affected by a number of abiotic stresses, such as extreme temperature and salt. Additionally, a variety of infective agents, such as downy mildew (DM), powdery mildew (PM), and root-knot nematodes (RKNs), can destroy the production of cucumber, as well as negatively affect other Cucurbitaceae species [29,30]. Therefore, it is quite necessary to excavate more potential resistance genes and define their functional roles in the stress resistance of cucumber. BBXs have been identified and functionally characterized in many plants, where they are generally encoded by a multigene family, such as *Arabidopsis thaliana* [3], *Oryza sativa* [31], *Solanum tuberosum* [32], *Malus domestica* [33], *Dendrobium officinale* [34], *Phyllostachys edulis* [35], *Vitis vinifera* [36–38], and *Capsicum annuum* [39,40]. In addition, a recent study identified 26 cucumber *BBX* genes and found that some of them are associated with fruit development and carotenoid biosynthesis [41]. To date, little information is available on the cucumber *BBX* genes in responses to various environmental stimuli. In this work, we carried out a genome-wide analysis of *BBX* genes in cucumber according to the cucumber genome v3 (an updated version in the cucumber 'Chinese long 9930' genome), including their gene and protein sequences, conserved motifs, gene duplication events, and *cis*-acting elements. Additionally, the expression profiles of the cucumber *BBX* genes under abiotic stresses (heat, cold, and salt) and biotic stresses (DM,

PM, and RKN) were also investigated. The results provide a biological basis for illustrating the functions of the *BBX* genes in the abiotic and biotic stress response of cucumber.

2. Materials and Methods

2.1. Identification and Characterization of BBX Family Members in Cucumber

To identify and annotate the BBX family members in cucumber, a search was firstly conducted against the cucumber genomic database v3.0 (with "9930" as the reference genome, http://cucurbitgenomics.org/, accessed on 20 May 2022) using the hidden Markov model (HMM) profile of the B-box domain (PF00643) with the HMMER software. Moreover, the *Arabidopsis* BBX proteins from the TAIR database (https://www.arabidopsis.org/, accessed on 20 May 2022) were used as the query sequences to search against the cucumber genomic database with blastp program (the cutoff value was 1×10^{-5}). Then, all putative BBX protein sequences were sent to PFAM (http://pfam.xfam.org/, accessed on 20 May 2022), SMART (http://smart.emblheidelberg.de/, accessed on 20 May 2022), as well as InterproScan (https://www.ebi.ac.uk/interpro/search/, accessed on 20 May 2022) online tools to guarantee the presence of the complete BBX domain. Identified CsBBX proteins were first subjected to the ProtParam website (http://www.expasy.org/, accessed on 20 May 2022) to calculate the molecular weight (MW), isoelectric point (pI), and grand average of hydropathicity (GRAVY) values, and their subcellular localizations were subsequently predicted with the Plant-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 20 May 2022).

2.2. Multiple Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment of cucumber BBX proteins was carried out by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on 20 May 2022) with default settings, and the results were displayed with the GeneDoc software. To study the evolutionary relationships, full-length BBX protein sequences from cucumber, grape, pear, *Arabidopsis*, and rice were first aligned using MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/, accessed on 20 May 2022) with default parameters, and then the comparison results were uploaded to Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software to construct the phylogenetic tree with the following parameters: Poisson correction, pairwise deletion, and a bootstrap test with 1000 replicates.

2.3. Gene Structure and Conserved Motif Analysis

The coding region sequence (CDS) and genomic DNA (gDNA) sequences were downloaded from the cucumber genomic database and submitted to GSDS online software (http://gsds.gao-lab.org/, accessed on 20 May 2022) to analyze the gene structure of each *CsBBX* gene. The Multiple Expectation Maximization for Motif Elicitation (MEME) (http://meme-suite.org/tools/meme, accessed on 20 May 2022) was used to examine the conserved motif arrangements of CsBBX proteins. The maximum number and minimum motif width of different motifs was set to 10 and 6, respectively.

2.4. Chromosomal Distribution, Gene Duplication, and Cis-Acting Elements Analysis of CsBBX Genes

The positional information of *CsBBX* genes was retrieved from the cucumber genomic database, and the MG2C (http://mg2c.iask.in/mg2c_v2.1/, accessed on 20 May 2022) online tool was employed to determine the chromosomal distributions of *CsBBX* genes. The Multiple Collinearity Scan toolkit (MCScanX) software was used to analyze the tandem and segment duplication events of *CsBBX* genes within the cucumber genome. To investigate the distribution of *cis*-acting elements within the promoter regions of *CsBBX* genes, the 2.0 kb upstream promoter sequence of the start codon (ATG) of each *CsBBX* gene was extracted and submitted to the PlantCARE server (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 20 May 2022).

2.5. Expression Profiling of the CsBBX Genes via RNA-Seq Data

To determine the tissue-specific expression of *CsBBX* genes, the RNA-seq data of various tissues at different developmental stages were retrieved from the Sequence Read Archive (SRA) database in NCBI (accession number: PRJNA80169). For the determination of the expression patterns of *CsBBX* genes in response to diverse abiotic stresses, the RNA-seq data were downloaded using the published RNA-seq dataset under heat stress (0, 3, and 6 h under 42 °C treatment; accession number: PRJNA634519) [42] and salt stress (leaves and roots of the seedlings after salt treatment under the accession numbers of PRJNA477930 and PRJNA511946; CK-L and Na-L, and CK-R and Na-R were the control and salt-stressed samples of leaves and roots, respectively) [43,44]. The RNA-seq datasets under diverse biotic stresses were also downloaded, including powdery mildew (PM, the PM susceptible cultivar D8 and PM resistant cultivar SSL508-28 at 0 h and 48 h after inoculation with the PM pathogen, accession number: PRJNA321023) [45], downy mildew (DM, the DM-susceptible cultivar Vlaspik and DM-resistant cultivar PI 197088 mock inoculated or infected with *Pseudoperonospora cubensis* at 1, 2, 3, 4, and 6 dpi, accession number: PRJNA285071) [46], and root-knot nematode (RKN, the RKN-susceptible line CC3 and RKN-resistant line IL10-1 at 0, 1, 2, and 3 dpi after infection with *Meloidogyne incognita*, accession number: SRP125669) [47], to analyze the expression patterns of the *CsBBX* genes under biotic stresses. The relative expression levels of *CsBBX* genes were calculated and normalized as transcripts per kilobase million (TPM) values for further analysis. These results were represented as heat maps using TBtools [30]. The *CsBBX* genes with expression values at a threshold of $|\log_2 (\text{fold change})| \geq 1$ and $p < 0.05$ were defined as differentially expressed.

2.6. Plant Materials, Abiotic Stress Treatment, and qRT-PCR Analysis

Two week old seedlings of cucumber variety '9930' were treated with cold and salt stress treatments following the methods described in our previous report [30]. Briefly, 2 week old seedlings were incubated with 1/2 Hoagland nutrient solution containing 200 mM NaCl or transferred to 4 °C for salt stress or cold stress, respectively. The leaves were collected respectively for three biological replicates at 0, 6, 12, and 24 h after the application of each treatment. Total RNA was extracted with the Trizol reagent, and then cDNA was reverse-transcribed using random primers. The quantitative real-time PCR (qRT-PCR) experiment was conducted with TB Green Premix Ex TaqII Kit (TaKaRa Biotechnology, Dalian, China) on a Roche Lightcyler 480II PCR System in triplicate according to the previously described method [48]. The $2^{-\Delta\Delta Ct}$ method was used to analyze the qRT-PCR results, and the primer sequences are provided in Table S1.

3. Results

3.1. Identification and Characterization of CsBBX Genes in Cucumber

To identify the *CsBBX* genes in cucumber, sequence homology analysis and protein domain validation were performed. As a result, only 22 genes encoding the corresponding conserved BBX domain were obtained, which were designated as *CsBBX1*–*CsBBX22* on the basis of their position on chromosomes 1–7 (Table 1). A comparison of the *BBX* genes with the published cucumber genomes is presented in Table S2. The CDS length of the *CsBBX* genes ranged from 399 bp (*CsBBX22*) to 1476 bp (*CsBBX10*). The CsBBX proteins contained 132 to 491 amino acids, and their molecular weight (MW) ranged from 14.63 kDa (CsBBX22) to 54.84 kDa (CsBBX15). The theoretical isoelectric point (pI) of the CsBBX proteins ranged from 4.31 (CsBBX9) to 8.40 (CsBBX8). The GRAVY scores of all CsBBX proteins were negative (ranged from −0.949 to −0.041), indicating that all CsBBX proteins are hydrophilic. According to the predicted subcellular localization results, all CsBBX proteins are located in the nucleus.

Table 1. Identification and characterization of *BBX* gene family in cucumber.

Gene	Accession No. (v3)	Chromosome: Location	Protein						Group
			CDS/bp	AA	pI	MW/kDa	GRAVY	Subcellular Localization	
CsBBX1	CsaV3_1G003800	chr1: 2,364,830–2,367,233	1083	360	5.25	41.63	−0.949	Nucleus	III
CsBBX2	CsaV3_1G011690	chr1: 7,244,708–7,246,426	795	264	5.29	29.49	−0.316	Nucleus	V
CsBBX3	CsaV3_1G031230	chr1: 18,437,778–18,448,748	1134	377	5.85	41.24	−0.308	Nucleus	I
CsBBX4	CsaV3_2G008220	chr2: 4,503,501–4,505,228	960	319	8.20	35.16	−0.395	Nucleus	I
CsBBX5	CsaV3_2G015160	chr2: 12,644,521–12,645,374	543	180	6.64	19.76	−0.301	Nucleus	IV
CsBBX6	CsaV3_2G029230	chr2: 19,152,113–19,154,409	921	306	6.65	32.67	−0.221	Nucleus	IV
CsBBX7	CsaV3_2G032640	chr2: 21,557,067–21,559,996	1014	337	6.08	36.93	−0.505	Nucleus	I
CsBBX8	CsaV3_2G035230	chr2: 23,569,718–23,570,660	402	133	8.40	14.66	−0.041	Nucleus	V
CsBBX9	CsaV3_3G044220	chr3: 36,085,222–36,088,641	825	274	4.31	30.15	−0.829	Nucleus	V
CsBBX10	CsaV3_4G002870	chr4: 1,790,472–1,794,059	1476	491	5.50	54.36	−0.545	Nucleus	II
CsBBX11	CsaV3_4G005810	chr4: 3,819,528–3,822,914	561	186	6.44	20.76	−0.605	Nucleus	IV
CsBBX12	CsaV3_4G008210	chr4: 5,753,161–5,758,556	894	297	5.31	32.30	−0.384	Nucleus	IV
CsBBX13	CsaV3_4G009980	chr4: 7,751,656–7,755,202	1035	344	5.24	38.47	−0.772	Nucleus	III
CsBBX14	CsaV3_5G034320	chr5: 27,321,989–27,323,306	1212	403	5.55	45.47	−0.710	Nucleus	III
CsBBX15	CsaV3_5G034710	chr5: 27,519,572–27,522,236	1464	487	6.81	54.84	−0.614	Nucleus	II
CsBBX16	CsaV3_6G003540	chr6: 2,826,773–2,829,268	1005	334	8.33	38.40	−0.823	Nucleus	III
CsBBX17	CsaV3_6G006790	chr6: 5,555,910–5,558,007	714	237	4.89	26.09	−0.285	Nucleus	IV
CsBBX18	CsaV3_6G009750	chr6: 7,889,202–7,896,870	1248	415	5.14	45.47	−0.481	Nucleus	II
CsBBX19	CsaV3_6G046900	chr6: 27,677,337–27,679,797	507	168	6.59	18.86	−0.567	Nucleus	IV
CsBBX20	CsaV3_7G000270	chr7: 395,757–397,689	1050	349	5.91	39.33	−0.359	Nucleus	IV
CsBBX21	CsaV3_7G002350	chr7: 1,863,318–1,866,691	1224	407	5.42	44.56	−0.520	Nucleus	II
CsBBX22	CsaV3_7G003780	chr7: 2,810,583–2,811,446	399	132	6.40	14.63	−0.242	Nucleus	V

3.2. Phylogenetic Analysis of BBX Family Members

To reveal the evolutionary relationship of the BBX proteins in cucumber and other plants, a phylogenetic tree involving the full-length amino-acid sequences of BBX proteins from cucumber, grape [37], pear [49], *Arabidopsis* [50], and rice [31] was constructed by MEGA 7.0. Phylogenetic analysis revealed that these BBX family members can be divided into five clades (I–V) (Figure 1), which was in accordance with the results in previous studies [12,51]. For the 22 CsBBX proteins, clade IV had the largest number of members (seven), followed by clades V, I, II, and III, which had five, four, three, and three members, respectively (Figure 1). Nearly all BBX proteins fell into the five clades on the basis of the number of B-box and CCT domains, except for CsBBX13 and CsBBX15, which were not categorized as expected. CsBBX13 was located in clade I but had one B-box and one CCT domain, while CsBBX15 was located in clade V but had two B-box domains and one CCT domain (Figures 1 and S1).

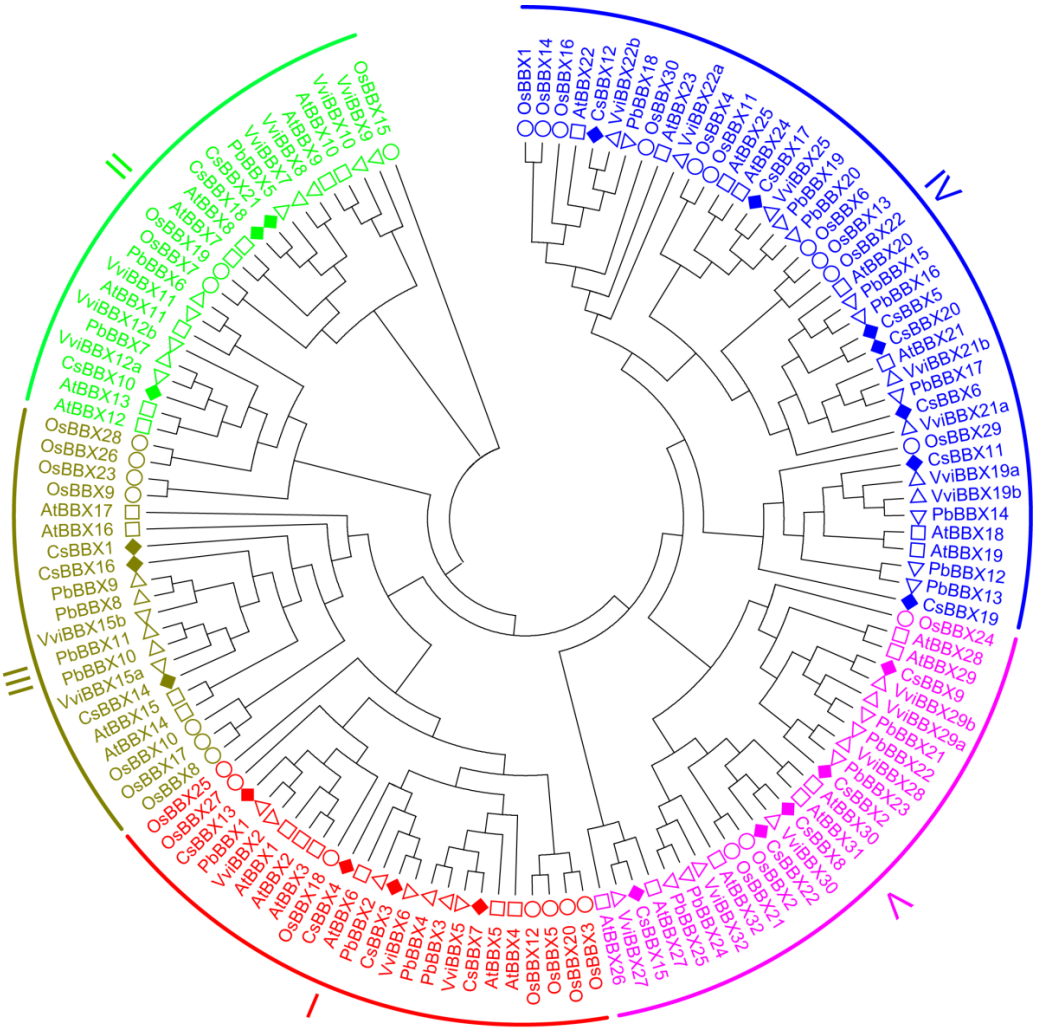

Figure 1. Phylogenetic tree analysis of BBX protein sequences. The full-length BBX protein sequences from *Arabidopsis*, rice, grape, pear, and cucumber were used to construct the phylogenetic tree with the MAFFT and MEGA 7.0 software. AtBBX, OsBBX, VviBBX, PbBBX, and CsBBX represent the BBX family members of *Arabidopsis*, rice, grape, pear, and cucumber, respectively. The BBX proteins in the phylogenetic tree were divided into five clades (I–V) with different colors.

3.3. Protein Motif and Gene Structure Analysis of BBX Members in Cucumber

To understand the structural features of CsBBX proteins, we first created a phylogenetic tree of the CsBBX proteins (Figures 2A and S1). The CsBBX proteins were clustered into five groups (I–V) on the basis of the diversity of their conserved domains following the previous study [3]. All CsBBX proteins had the conserved B-box signature sequence $CX_2CX_8CX_7X_2CDX_3H$ with conserved cysteine (Cys), histidine (His), and aspartic acid (Asp) (Figures 2A and S1), among which the Asp residue is considered to be involved in transcriptional activation and DNA binding for BBX proteins [14,34].

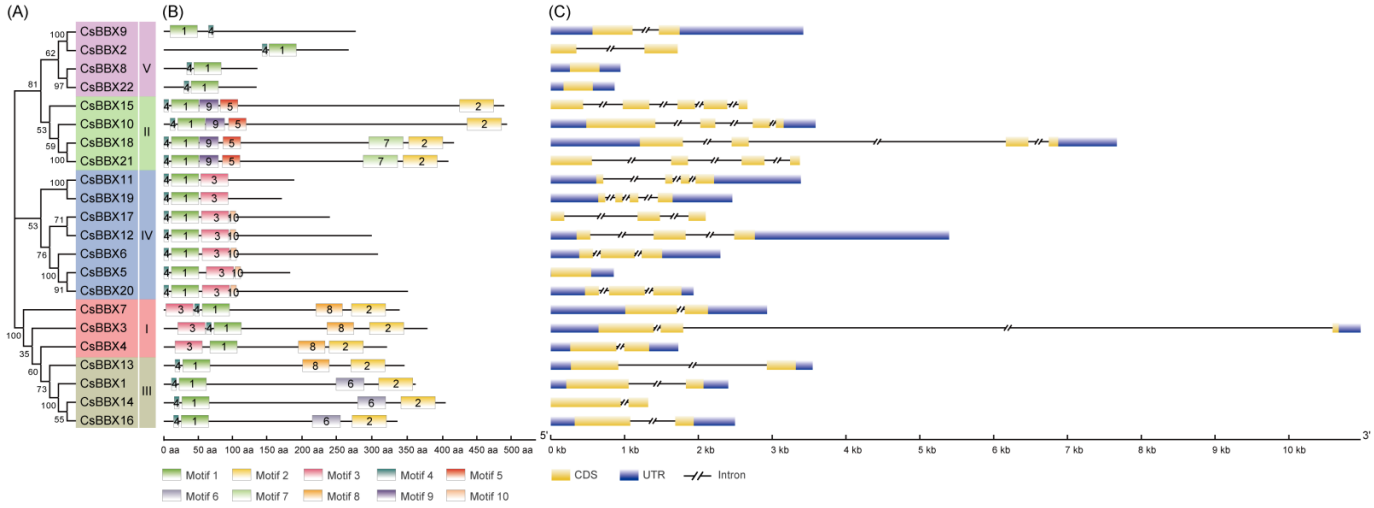

Figure 2. Phylogenetic tree (**A**), conserved motif arrangement (**B**), and gene structure diagram (**C**) of *BBX* gene family in cucumber. (**A**) The NJ phylogenetic tree was created using the MEGA 7.0 program with 1000 bootstrap repeats, and CsBBX proteins in the phylogenetic tree were divided into five groups (I–V) with different colors. (**B**) Distribution of conserved motifs in CsBBX proteins identified by MEME. The length and name of conserved motifs are represented by boxes with different colors. (**C**) Gene architecture of the *CsBBX* genes. The yellow and blue boxes represent the CDSs and UTRs, respectively, while the black lines show the introns.

Subsequently, we analyzed the conserved motifs of CsBBX proteins by MEME and predicted a total of 10 distinct motifs (designated as motifs 1–10). Among them, motif 9 and motif 5 constituted the BBX domain; motif 1 and motif 3 were also annotated as the BBX domain, while motif 2 was associated with the CCT domain (Figure 2B). All CsBBXs had motif 1 and motif 4, with the exception of CsBBX4. Motif 2 was exclusively present in the CsBBXs of group I–III, while motif 6, motif 7, and motif 10 were only found in the CsBBXs of group III, II, and IV, respectively. In addition, all the CsBBXs in group I and CsBBX13 from another group contained motif 8, while motif 5 and motif 9 were exclusively present in the CsBBXs of group II (Figure 2B).

To explore the structural diversity of *CsBBX* genes, we compared the CDS and corresponding gDNA sequence to illustrate a gene structural diagram (Figure 2C). The results showed that the *CsBBX* genes had 0–4 introns, and most of them contained 1–3 introns. Amongst them, *CsBBX15* had the largest number of introns, whereas three *CsBBX* genes (*CsBBX5*, *CsBBX8*, and *CsBBX22*) were found to be intronless. Notably, all *CsBBX* genes in group III possessed only one intron, while the *CsBBX* genes in group II and group IV had 2–4 introns (except for *CsBBX5*) (Figure 2C).

3.4. Chromosomal Location and Gene Duplication of the CsBBX Genes

To visualize the location of *CsBBX* genes on chromosomes, their annotations and positions were downloaded and visualized by MG2C online software. As shown in Figure 3, all *CsBBX* genes were unevenly located at seven chromosomes of cucumber. Chromosome 3 and chromosome 2 had the smallest (one) and largest (five) number of *CsBBX* genes,

respectively. Chromosomes 4 and 6 contained four *CsBBX* genes; chromosomes 1 and 7 were observed to each have only three *CsBBX* genes, followed by chromosome 5, which contained two *CsBBX* genes. In addition, analysis of gene duplication events indicated that a total of nine pairs of *CsBBX* genes underwent segmental duplication (Figure 3).

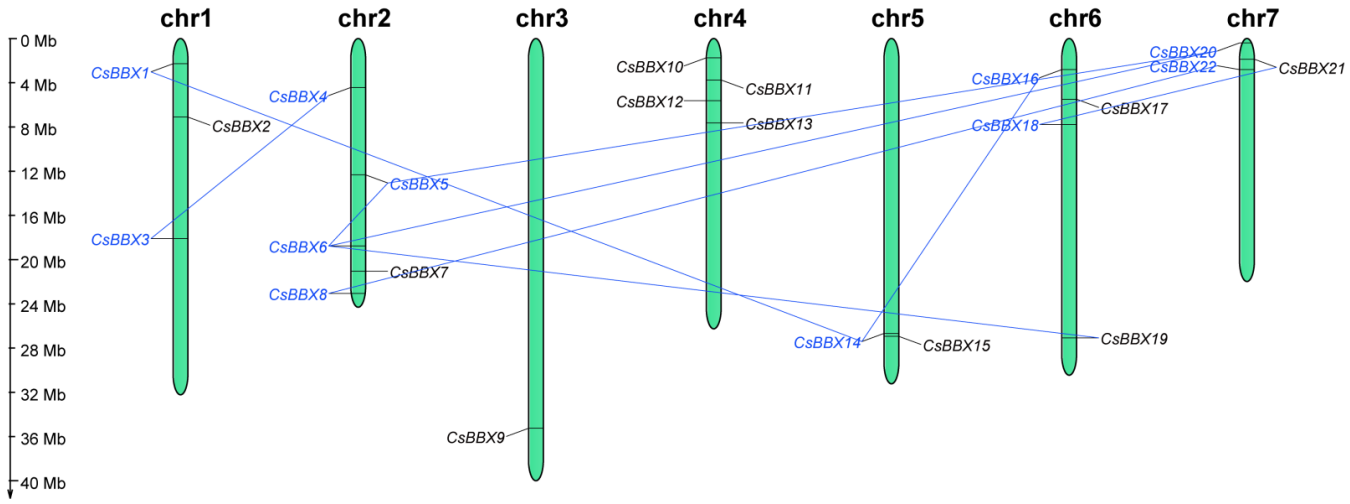

Figure 3. Distribution of the *CsBBX* genes on seven cucumber chromosomes. The scale on the left represents megabases (Mb). The segmental duplication genes are marked with blue lines.

3.5. Cis-Acting Elements in the Promoter Regions of the CsBBX Genes

To determine the potential biological function of *CsBBX* genes, the PlantCARE server was used to analyze their promoter regions. By screening the function of *cis*-acting elements, we found 13 and nine types of *cis*-acting elements associated with hormone and stress responses, respectively (Figure 4). For the hormone-related *cis*-acting elements, ABRE (involved in abscisic acid (ABA) response) was the most abundant *cis*-element in *CsBBX* genes, followed by gibberellin-related elements (TATC-box, P-box, and GARE-motif), and then auxin-responsive elements (AuxRE, TGA-box, AuxRR-core, and TGA-element). Notably, the *CsBBX9* gene had 11 ABRE elements in its promoter region, implying that *CsBBX9* may play a key role in ABA response. In terms of elements associated with MeJA (CGTCA-motif and TGACG-motif) and salicylic acid (TCA-element), they were widely distributed in 17 and 12 *CsBBX* genes, respectively. In addition, the *CsBBX4* gene had more *cis*-elements related to MeJA than other *CsBBX* genes, indicating that it may function in MeJA response. As for stress-related *cis*-elements, all the *CsBBX* genes had one to six AREs (related to anaerobic induction) with the exception of *CsBBX10* and *CsBBX22*. Moreover, the GC-motif (related to anoxic specific inducibility) was only present in *CsBBX19*, and there were three MBS elements (involved in drought response) in *CsBBX14*. As for the STRE element (involved in stress response), it was widely present in most *CsBBX* genes (18 out of 22) (Figure 4), suggesting that the *CsBBX* genes may participate in multiple stress responses.

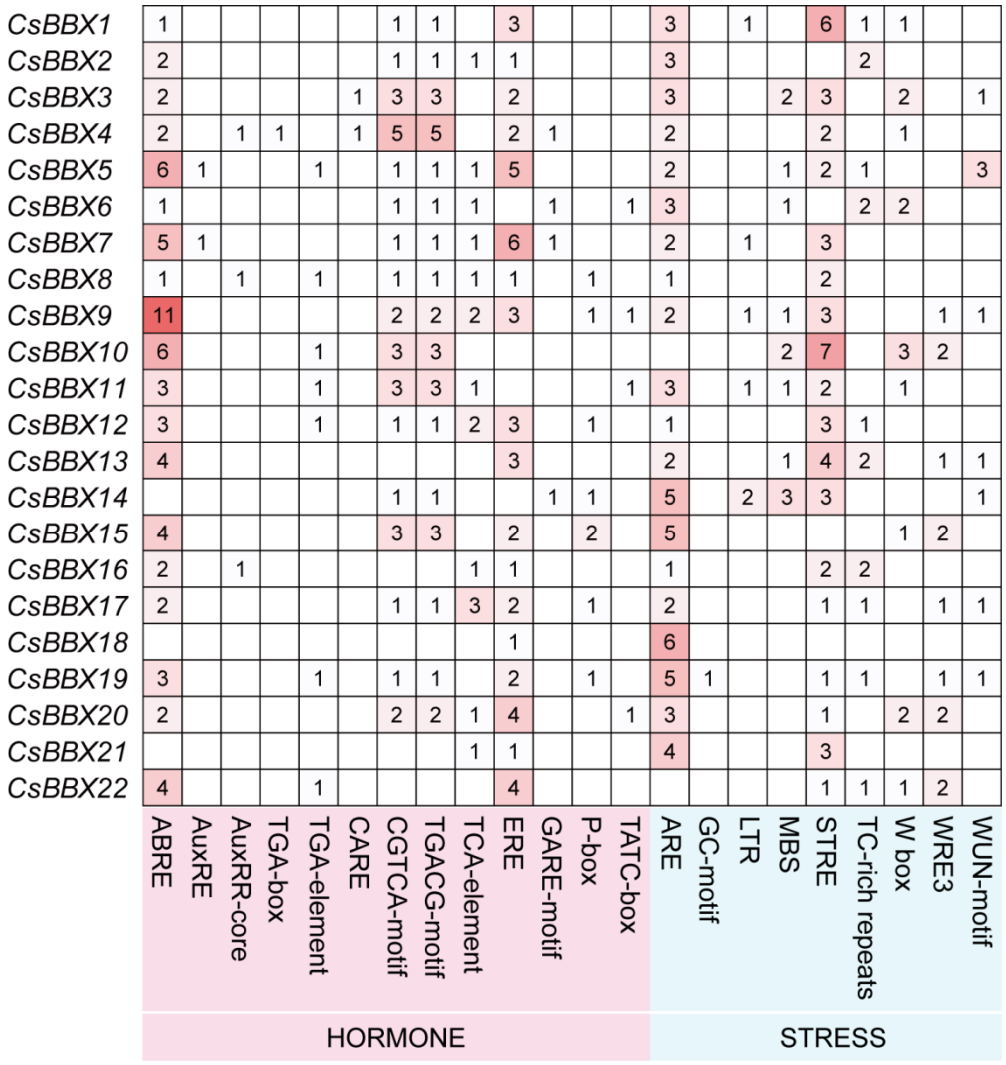

Figure 4. Types and numbers of hormone- and stress-related *cis*-elements in *CsBBX* promoter regions.

3.6. Expression Patterns of CsBBX Genes in Different Tissues of Cucumber

To investigate the potential roles of *CsBBX* genes in plant growth and development, the tissue expression patterns of 22 *CsBBX* genes were analyzed according to the available RNA-seq data. As shown in Figure 5, all *CsBBX* genes had expression in at least one tested tissue, with *CsBBX3*, *CsBBX9*, *CsBBX10*, *CsBBX12*, *CsBBX18*, *CsBBX20*, and *CsBBX21* having high expression in all tested tissues. Two *CsBBX* genes (*CsBBX2* and *CsBBX22*) and one *CsBBX* gene (*CsBBX13*) were exclusively expressed in the root and tendril, respectively, implying that they may play a critical role in the corresponding tissues. Furthermore, *CsBBX6*, *CsBBX7*, *CsBBX16*, and *CsBBX21* were more abundantly expressed in the root, stem, leaf, and flower, respectively, while four *CsBBX* genes (*CsBBX8*, *CsBBX11*, *CsBBX12*, and *CsBBX19*) showed the highest expression in the tendril. Interestingly, 10 *CsBBX* genes (*CsBBX1*, *CsBBX3*, *CsBBX4*, *CsBBX6*, *CsBBX10*, *CsBBX13*, *CsBBX15*, *CsBBX16*, *CsBBX17*, and *CsBBX22*) exhibited relatively higher expression levels in unfertilized ovaries than in unexpanded ovaries and fertilized ovaries, indicating that they might play a role in ovary development (Figure 5).

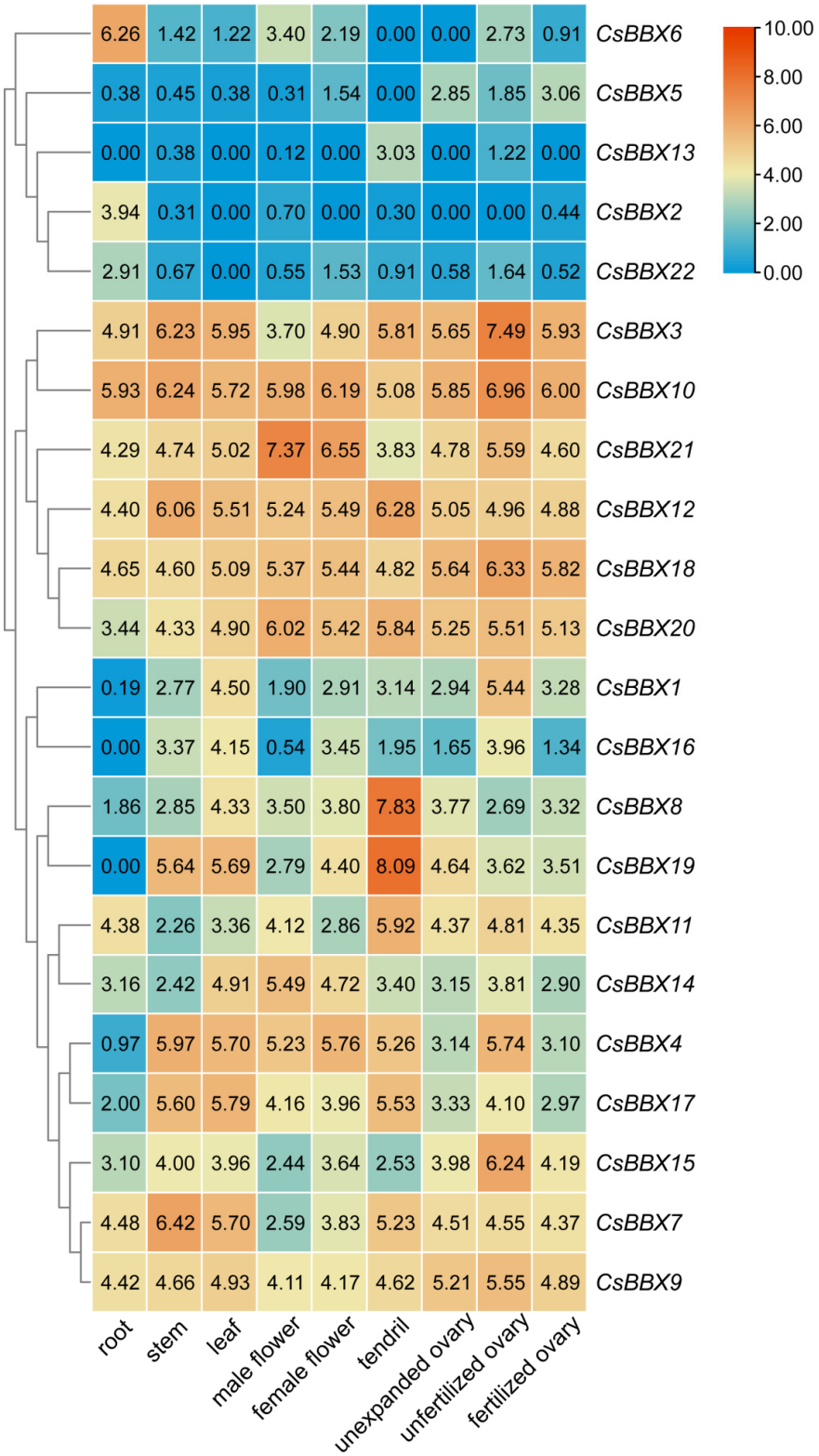

Figure 5. Expression levels of *CsBBX* genes in various cucumber tissues. The expression data were displayed and clustered with TBtools using $\log_2$-transformed TPM values ($\log_2$ (TPM + 1)).

3.7. Expression Patterns of CsBBX Genes under Multiple Abiotic Stresses

To investigate the functions of *CsBBX* genes in response to environmental stimuli, the expression patterns of *CsBBX* genes under heat and salt stresses were determined according to RNA-seq data from the public available transcriptional database. Upon heat stress, the

expression of *CsBBX1*, *CsBBX4*, *CsBBX14*, *CsBBX16*, *CsBBX19*, and *CsBBX20* decreased significantly compared with the control (0 h), while that of *CsBBX7*, *CsBBX17*, *CsBBX18*, and *CsBBX22* was significantly upregulated. Furthermore, the expression levels of *CsBBX9*, *CsBBX11*, and *CsBBX13* declined at 3 h, while they were induced at 6 h under heat stress (Figure 6A). The transcriptional levels of *CsBBX* genes in leaf and root samples of cucumber under salt stress are shown in Figure 6B. In leaves, the expression levels of *CsBBX8* and *CsBBX22* were significantly increased, while those of *CsBBX13* and *CsBBX18* were significantly inhibited under salt stress. In addition, salt stress obviously downregulated the expression levels of *CsBBX2*, *CsBBX5*, and *CsBBX13* in roots. These findings suggest that the *CsBBX* genes might play different roles in response to multiple abiotic stresses.

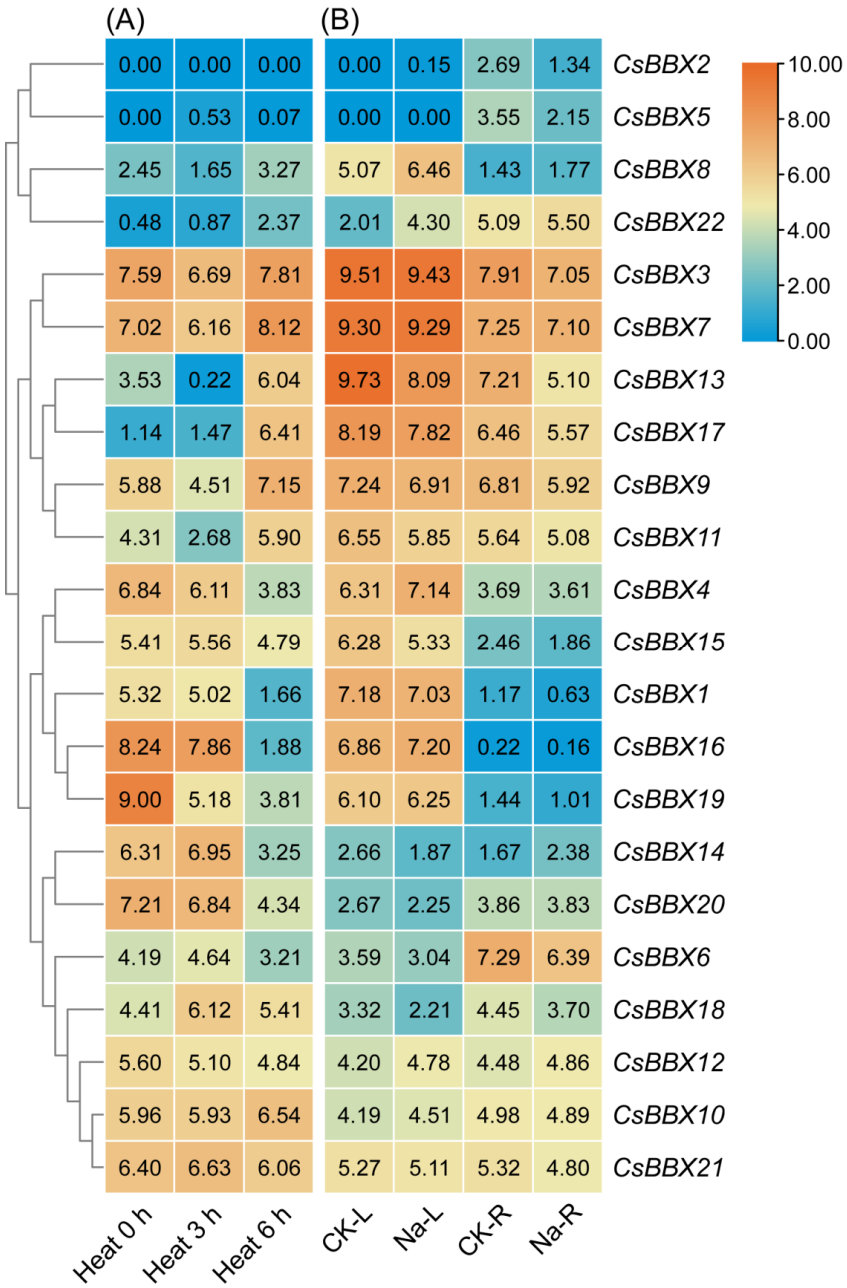

Figure 6. Expression patterns of the *CsBBX* genes in cucumber exposed to various abiotic stresses based on data retrieved from available RNA-seq data. (**A**) The expression heatmap of the *CsBBX* genes under heat stress at 0, 3, and 6 h. The data in the boxes indicate $\log_2$ (TPM + 1) values. (**B**) The expression heatmap of the *CsBBX* genes under salt stress. CK-L and Na-L, and CK-R and Na-R are the control and salt-stressed samples of leaves and roots, respectively. The data in the boxes indicate $\log_2$ (TPM + 1) values.

The transcriptional levels of six selected *CsBBX* genes under cold and salt stress were further determined by qRT-PCR. Under cold stress, the transcription of *CsBBX1* showed no significant change at all test time points, while other *CsBBX* genes displayed significant decreases in expression at certain time points. Amongst them, *CsBBX7* and *CsBBX10* were downregulated at the early time points (6 h or 12 h), followed by an increase at the late timepoint (24 h). However, *CsBBX8* showed quite different changes in transcription level, as its transcription was significantly increased at 6 h, and then decreased later (Figure 7). Under salt stress, the expression level of *CsBBX1* showed no significant change, while that of other *CsBBX* genes was significantly induced at certain time points (Figure 8).

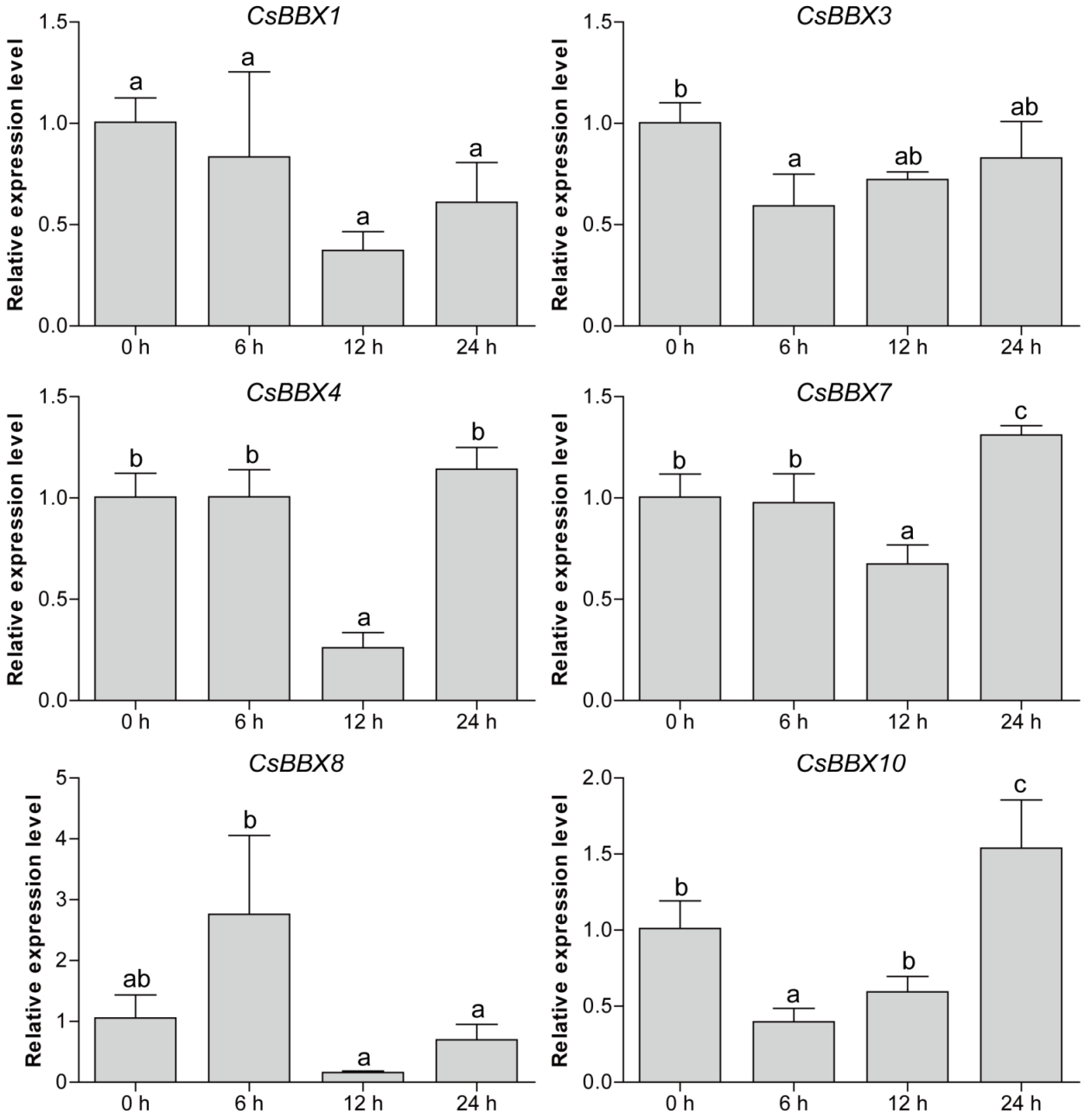

Figure 7. qRT-PCR analysis of six selected *CsBBX* genes under cold stress. The seedlings were kept at 4 °C for cold stress, and leaf samples were collected at 0, 6, 12, and 24 h. Data were normalized to the *CsAct3* gene expression level, and the value of each *CsBBX* gene at 0 h was normalized as "1.0". Different lowercase letters indicate significant differences among all the test data.

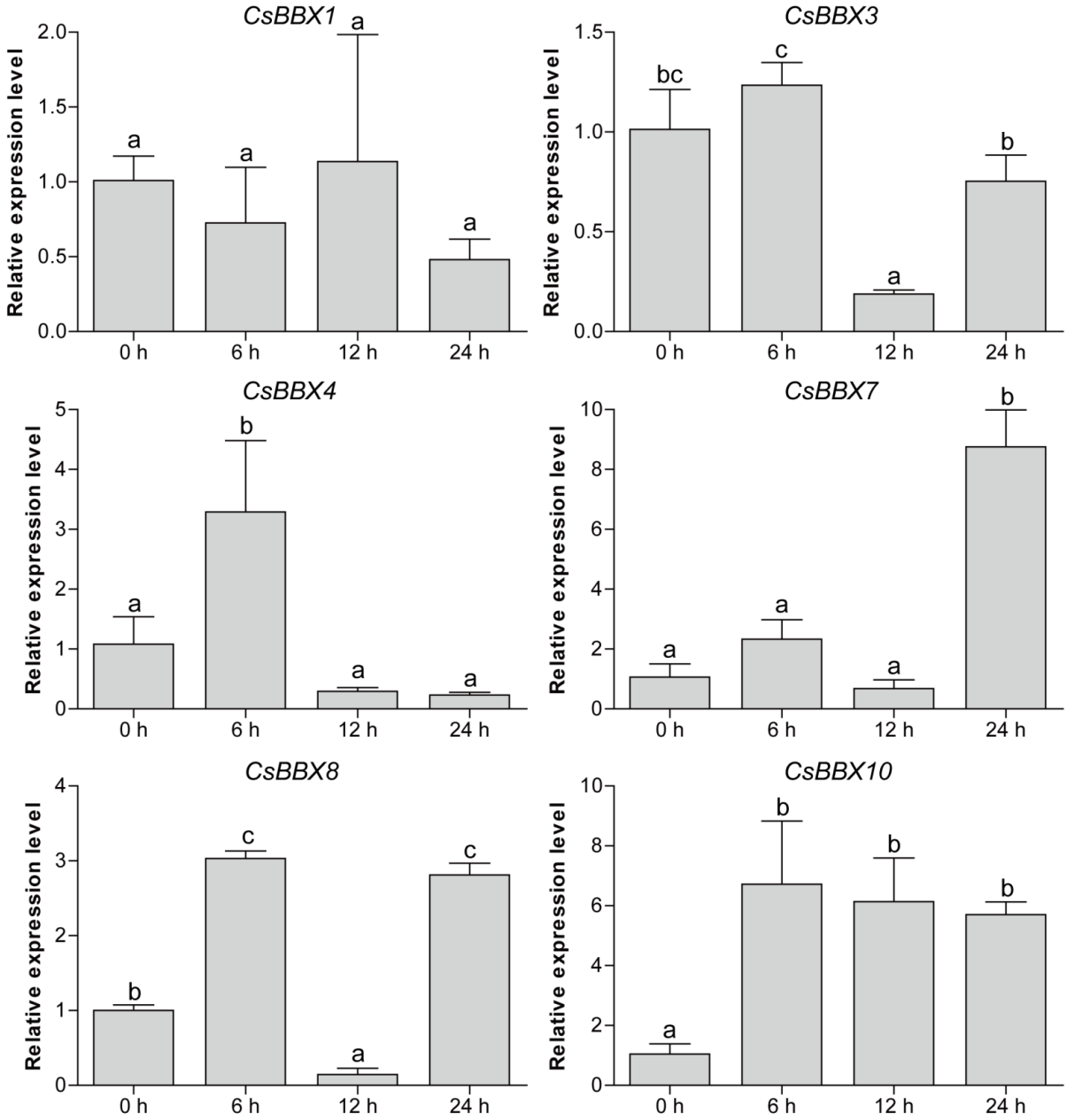

Figure 8. qRT-PCR analysis of six selected *CsBBX* genes under salt stress. The seedlings were treated with 200 mM NaCl for salt stress, and leaf samples were collected at 0, 6, 12, and 24 h. Data were normalized to the *CsAct3* gene expression level, and the value of each *CsBBX* gene at 0 h was normalized as "1.0". Different lowercase letters indicate significant differences among all the test data.

3.8. Expression Patterns of CsBBX Genes in Response to Biotic Stress

To unravel the role of *CsBBX* genes in response to biotic stress, the expression data of *CsBBX* genes under the inoculation with PM, DM, and RKN were downloaded. Under PM inoculation, a total of six and six *CsBBX* genes were differentially expressed in the D8 (PM-susceptible) and SSL508-28 (PM-resistant) cucumber lines, respectively (Figure 9A). In the PM-susceptible line, *CsBBX4*, *CsBBX7*, *CsBBX13*, and *CsBBX16* displayed downregulated expression, while *CsBBX22* was upregulated after PM treatment compared with the control. In the PM-resistant line, *CsBBX4*, *CsBBX7*, *CsBBX12*, and *CsBBX22* were obviously upregulated, whereas *CsBBX15* and *CsBBX16* were significantly downregulated after PM treatment (Figure 9A). Under DM inoculation, a total of 13 and 15 *CsBBX* genes were differentially expressed in the Vlaspik (DM-susceptible) and PI 197088 (DM-resistant) cucumber lines,

respectively. Amongst them, *CsBBX6*, *CsBBX11*, *CsBBX13*, and *CsBBX15* were downregulated over time in both sensitive and resistant cucumber plants, while two other *CsBBX* genes (*CsBBX4* and *CsBBX16*) were downregulated at the earlier timepoint (1 dpi), but their expression levels subsequently increased in both sensitive and resistant cucumber plants (Figure 9B). Compared with those in the control, *CsBBX7*, *CsBBX17*, and *CsBBX19* exhibited upregulated expression after DM treatment at certain time points in the DM-susceptible line, while *CsBBX4*, *CsBBX14*, *CsBBX18*, and *CsBBX19* showed upregulated expression at certain time points in the DM-resistant line (Figure 9B). Under RKN inoculation, a total of 10 genes (*CsBBX4*, *CsBBX7*, *CsBBX8*, *CsBBX9*, *CsBBX11*, *CsBBX13*, *CsBBX16*, *CsBBX17*, *CsBBX19*, and *CsBBX22*) were upregulated, and three genes (*CsBBX2*, *CsBBX5*, and *CsBBX14*) were downregulated in both sensitive and resistant cucumber plants (Figure 9C). However, the expression of *CsBBX3* was obviously increased after RKN treatment in CC3 (RKN-susceptible), whereas its expression showed no change in IL10-1 (RKN-resistant) cucumber plants (Figure 9C).

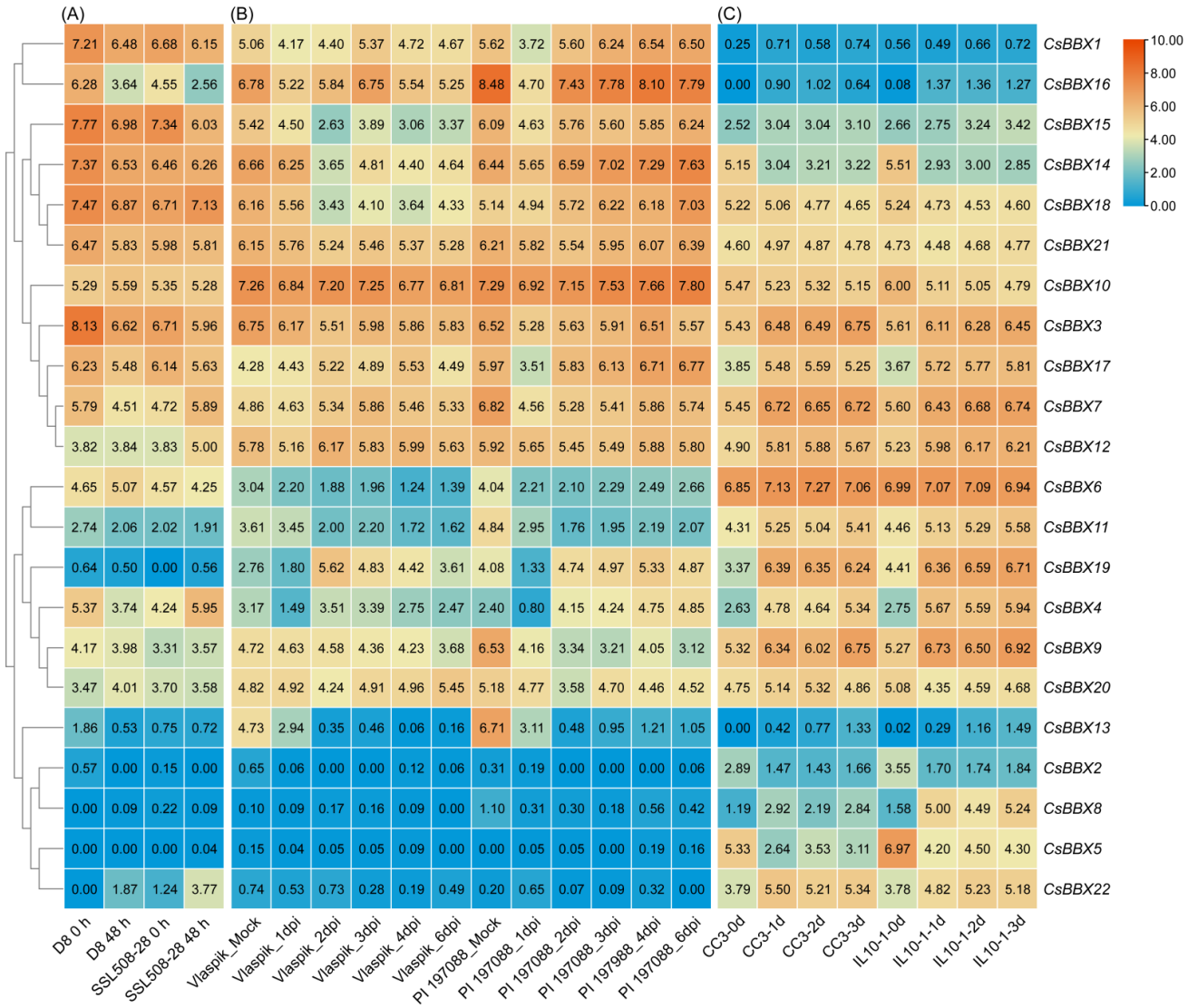

Figure 9. Expression profiles of *CsBBX* genes in cucumber inoculated with PM (**A**), DM (**B**), and RKN (**C**). (**A**) Expression of *CsBBX* genes in response to PM treatment in cucumber cultivars D8 (PM-susceptible) and SSL508-28 (PM-resistant). (**B**) Expression of *CsBBX* genes in Vlaspik (DM-susceptible) and PI 197088 (DM-resistant) cultivars of cucumber after DM inoculation. Mock, control sample. (**C**) Expression of *CsBBX* genes at different time points after infection with *M. incognita* in a RKN-susceptible line (CC3) and RKN-resistant line (IL10-1). The expression data were displayed and clustered with TBtools using $\log_2$ (TPM + 1) values; dpi, days after inoculation.

4. Discussion

Plant-specific TFs such as BBXs play crucial roles in different growth and development processes, as well as responses to diverse stresses [1]. In the current study, a comprehensive analysis of the cucumber *BBX* gene family was carried out on the basis of the updated version cucumber genome (v3), and a total of 22 *BBX* genes were identified (Table 1). The number of cucumber *BBX* genes is comparable to that in sorghum (24) [52], grape (25) [36,37], petunia (28) [53], rice (30) [31], tomato (31) [51], and *Arabidopsis* (32) [3], while the genome size varies greatly among these species. A recent study demonstrated that genome and segmental duplication play a significant role in the expansion of the *BBX* gene family [12]. In this study, only nine segmental duplication gene pairs were identified, and no tandem duplication was detected (Figure 3). Similar results were also reported in many other plants [12,49,52], suggesting that the *BBX* gene family in cucumber has been expanded, mainly attributed to segmental duplication.

According to the phylogenetic results of the BBX proteins from cucumber and four other plant species, the CsBBX proteins can also be divided into five clades, and each CsBBX has at least one homolog in these plant species (Figure 1), implying that their functions may be evolutionarily conserved. Protein motif and gene structure analysis revealed that the evolutionarily close CsBBXs exhibit strong conservation in gene architecture and domain organization, but *CsBBX* genes in group II and group IV tend to have more introns than those in other three groups (no more than two introns) (Figure 2). Both gain and loss of introns may occur during the evolution of genes within families, which may be responsible for their functional divergence. According to the hypothetical model of B-box domain evolution [12,54], it can be speculated that intron gain rather than intron loss has contributed to the different structures of *CsBBX* genes across different groups (particularly group II and group IV) during their evolution.

Promoters can provide directional support for the expression and possible role of genes in the interaction between plants and environment stimuli [29]. *CsBBX* promoters include a series of hormone- and stress-related *cis*-elements (Figure 4), which have also been observed in the promoters of the *BBX* genes in other plant species, such as *Solanum lycopersicum* [55], *Vitis vinifera* [38], *Gossypium hirsutum* [56], and *Brassica rapa* [57], and the *BBX* genes in these plants are regulated by diverse stress treatments. In the present study, six *CsBBX* genes displayed variations in expression under salt stress according to RNA-seq and qRT-PCR results (Figures 6B and 8), indicating that *CsBBX* genes might play crucial roles in response to salt stress. Wintersweet (*Chimonanthus praecox*) *CpBBX19* was upregulated by four types of abiotic stress (heat, cold, salt, and drought), and overexpression of *CpBBX19* promoted salt and drought stress tolerance in transgenic *Arabidopsis* plants [58]. In addition, qRT-PCR results showed that five out of the six selected *CsBBX* genes were differentially expressed under cold stress (Figure 7), suggesting that *CsBBX* genes might also participate in response to cold stress. A previous report showed that *SlBBX7*, *SlBBX9*, and *SlBBX20* positively participate in cold stress tolerance in tomato plants [51]. Apple MdBBX37 can fine-tune jasmonic acid (JA)-mediated cold stress tolerance through the MIEL1–BBX37–ICE1–CBF and JAZ–BBX37–ICE1–CBF pathways [59]. In particular, a total of 13 *CsBBX* genes were found to be responsive to heat stress, and seven *CsBBX* genes (*CsBBX7*, *CsBBX9*, *CsBBX11*, *CsBBX13*, *CsBBX17*, *CsBBX18*, and *CsBBX22*) were significantly upregulated after heat stress treatment (Figure 6A), implying that they may be the key regulators of heat tolerance in cucumber. In a recent study, *SlBBX17* was found to be induced by heat stress, and its overexpression increased the heat tolerance of tomato by modulating the expression of heat stress-responsive genes [26]. In *Arabidopsis*, BBX18 plays a vital role in high-temperature-induced hypocotyl growth by not only preventing PRR5 from suppressing PIF4 but also promoting the degradation of ELF3 [5,25]. *CsBBX11* and *CsBBX19* are close homologs of *BBX18* (Figure 1), both of which showed great variations in expression level in response to heat stress (Figure 6A), implying their possible roles in thermomorphogenesis.

Accumulating evidence has demonstrated that BBXs also play essential roles in regulating plant defense responses. For instance, the expression level of two group II *BBX*

genes, *OsCOL9* and *MaCOL1*, was significantly increased after the infection of *Magnaporthe oryza* and *Colletotrichum musae*, respectively [60]. In *Ipomoea trifida*, the expression of 14 and 10 *ItfBBX* genes was significantly upregulated under β-aminobutyric acid (BABA) and benzothiadiazole *S*-methyl ester (BTHT) treatments, respectively [61]. In the present study, a total of eight, 15, and 15 *CsBBX* genes displayed significant variations in expression level after PM, DM, and RKN inoculation, respectively (Figure 9). In addition, many *cis*-acting elements related to hormone, including JA and SA, were identified in most *CsBBX* genes (Figure 4), indicating that the *CsBBX* genes are involved in response to biotic stress and hormonal signal transduction pathways. In a previous study, the expression of 10 *VviBBX* genes was upregulated, while that of seven other *VviBBX* genes was downregulated under PM treatment in grape, and the *VviBBX* genes were also found to be regulated by multiple phytohormones to various degrees [37]. Notably, *CsBBX4* was differentially expressed upon the infection of all three phytopathogens in both susceptible and resistant varieties, suggesting that it may be a key regulator of the defense response to PM, DM, and RKN inoculation.

5. Conclusions

In conclusion, genome-wide identification and characterization of the *BBX* gene family were conducted in cucumber, and the phylogenetic relationship, protein and gene structures, and tissue expression patterns of the *CsBBX* genes were systematically analyzed. A total of 22 *CsBBX* genes were identified, and they were distributed across the seven cucumber chromosomes. Amongst them, 11 *CsBBX* genes made up to nine segmental duplication events, while no tandem duplication events were found. Simultaneously, our analysis found that the CsBBX proteins were clustered into five structural groups (I–V) according to the diversity of their conserved domains, and evolutionarily close CsBBXs exhibited strong conservation in gene structure and conserved motif organization. Furthermore, promoter analysis indicated that *CsBBX* genes contained many *cis*-elements that are responsive to various hormones and stresses, and the expression patterns of stress-responsive *CsBBX* genes were elucidated on the basis of RNA-seq and qRT-PCR results. These findings provide useful information for further functional characterization of *CsBBX* genes in cucumber growth regulation and stress response, further laying a foundation for cucumber crop improvement.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture12060827/s1: Figure S1. Amino-acid sequence conservation within the B-box domains of cucumber BBX proteins; Table S1. The gene-specific primers used for qRT-PCR; Table S2. List of the cucumber *BBX* genes identified in this study.

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