

Article

Genome-Wide Identification and Expression Analysis of the *ZjBBX* Gene Family and Its Potential Role in Response to Fruit Coloring, Salt Stress Response, and Jujube Witches' Broom in Chinese Jujube (*Ziziphus jujuba* Mill.)

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Abstract

BBX transcription factors are key regulators of plant growth, development, and stress responses, yet their functions in jujube (*Ziziphus jujuba* Mill.), an economically important fruit tree, remain largely unexplored. Here, we systematically identified 26 *ZjBBX* genes and comprehensively analyzed their phylogenetic relationships, structural features, and expression dynamics. Our integrative approach revealed three major findings. First, nine *ZjBBX* genes exhibited distinct expression patterns during fruit coloration, with *ZjBBX9* and *ZjBBX25* emerging as persistent up-regulated candidates. Second, under salt stress, *ZjBBX* members displayed three contrasting response modes, sustained down-regulation, rapid induction, or sharp repression, suggesting functional diversification in salinity adaptation. Third, under jujube witches' broom, *ZjBBX8*, *ZjBBX11*, and *ZjBBX13* showed differential expression between healthy and infected materials, implicating them in biotic stress defense. Collectively, this study provides the first multidimensional expression atlas of the *ZjBBX* family linked to fruit quality, salt tolerance, and pathogen resistance in jujube. Our findings open new avenues for marker-assisted breeding and genetic improvement of stress-resilient jujube cultivars.

Keywords: expression analysis; fruit color; jujube witches broom; salt stress; *ZjBBX* gene family; *Ziziphus jujuba* Mill.



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1. Introduction

Plants, as sessile organisms, cannot actively avoid unfavorable environments. To coordinate growth, development, and environmental adaptation, they have evolved complex transcriptional regulatory networks. Among these, B-box (BBX) proteins constitute a family of transcription factors characterized by zinc-finger domains. Their N-terminal region typically contains one or two tandem B-box domains, whereas some members also possess

C-terminal CCT domains and/or VP motifs, which mediate protein–protein interactions, DNA binding, and nuclear localization [1,2]. Based on structural differences, plant BBX proteins are classified into five distinct groups [1]. BBX family members participate in light signaling, circadian rhythm regulation, and stress responses by interacting with key regulators such as HY5, COP1, and PIFs, thereby modulating seed germination, flowering, plant architecture, pigment accumulation, and defense processes [3–7]. Although several reviews have addressed the roles of BBX proteins in photomorphogenesis, flowering control, and abiotic stress responses [3,8–13], further investigation is still warranted.

Beyond these functions, BBX proteins also play critical roles in biotic stress responses, often through crosstalk with hormone signaling pathways, including salicylic acid (SA) and jasmonic acid (JA). For example, in *Capsicum annuum* ‘Hangjiao No. 12’, *CaBBX14* expression was significantly upregulated upon *Phytophthora capsici* infection or exogenous SA application. Silencing *CaBBX14* compromised pepper resistance to *P. capsici*, accompanied by reduced SA levels and suppressed expression of SA-related defense genes, suggesting that *CaBBX14* positively regulates SA-mediated disease resistance [14]. In sweet potato (*Ipomoea batatas*), *IbBBX24* is involved in the JA-mediated response to *Fusarium wilt* through binding to the promoters of *IbJAZ10* and *IbMYC2* [15]. Additionally, several BvBBX genes in sugar beet (*Beta vulgaris* L.) showed differential expression upon *Cercospora* leaf spot infection [16]. These findings highlight the considerable regulatory importance of BBX transcription factors in plant immunity.

The jujube tree (*Ziziphus jujuba* Mill.) is an important fruit species native to China and is now cultivated in more than 48 countries worldwide. It exhibits remarkable tolerance to drought and saline-alkali conditions, making it a valuable economic resource for approximately 20 million farmers in arid and semi-arid regions [17,18]. However, the composition of BBX members in jujube remains poorly understood. Moreover, jujube production is seriously threatened by jujube witches’ broom (JWB), a severe biotic disease caused by phytoplasma. This disease is highly contagious, difficult to cure, and has a high fatality rate; infected trees usually die within about three years, with a mortality rate approaching 100% [19,20]. Nevertheless, the involvement of BBX transcription factors in response to JWB has not yet been elucidated.

Despite the economic importance of jujube (*Ziziphus jujuba*), the BBX gene family—known for its regulatory roles in plant growth and stress responses—has not yet been characterized in this species. Moreover, the molecular mechanisms underlying jujube witches’ broom, a devastating phytoplasma-associated disorder, remain largely unclear. To investigate the role of the *ZjBBX* genes in fruit coloring and responses to abiotic stress and diseases, this study aims to systematically identify and characterize the BBX family in the jujube T2T genome, and investigate their expression dynamics in response to witches’ broom infection. Through integrative phylogenomic and transcriptomic analyses, we identified candidate *ZjBBX* genes associated with disease response and provided a foundational resource for functional validation. These findings not only facilitate future mechanistic studies of *ZjBBX*-mediated fruit coloring and stress regulation but also offer potential targets for molecular breeding aimed at enhancing disease resistance in jujube.

2. Materials and Methods

2.1. Plant Materials and Treatment

For fruit coloration analysis, the red jujube cultivar ‘Jinsi Xiaozao’ (JS) was selected. Fruit samples were collected at five developmental stages: white ripening (F5, 75 days after full bloom), late white ripening (F6, 85 days after full bloom), quarter-red (F7, 95 days after full bloom), semi-red (F8, 105 days after full bloom), and full-red (F9, 120 days after full bloom) [21].

Seeds of sour jujube were sown in seedling pots and grown in a controlled-environment growth chamber under a 12 h light/12 h dark photoperiod, with a constant temperature of 23–25 °C and relative humidity of 70%. At the seven- to nine-leaf stage, healthy and uniformly sized seedlings were selected and divided into two treatment groups: a control (CK, irrigated with water) and a salt-stress group (Na, treated with 400 mmol/L NaCl, as this was identified in existing research reports. Compared to the CK, 200 mmol/L NaCl treatment could not induce the damage within 8 d. However, under 400 mmol/L NaCl treatment, the leaves of sour jujube seedlings appeared yellowing, curling, and gradually wilting within 8 d compared to the control). Each treatment consisted of three biological replicates [22].

In this study, the susceptible genotype ‘Fu’ and the tolerant genotype ‘T13’ of jujube (*Ziziphus jujuba*) were used. The experiment was conducted at the Jujube Experimental Base of Hebei Agricultural University in Fuping. In May, scions from both genotypes were grafted onto ‘Fu’ rootstocks that had been infected with phytoplasma. As negative controls, scions from the same genotypes were also grafted onto healthy ‘Jiang’ rootstocks free of phytoplasma [23].

2.2. Identification and Physicochemical Properties of the Jujube BBX Genes

The complete genome sequence, protein sequence, and annotation files of jujube were retrieved from the Jujube Genome Factory. The genome assembly (ASM3175591v1) [24] was used to identify BBX candidate genes through two complementary approaches. First, the hidden Markov model (HMM) profile of the BBX domain (PF00643) was downloaded from InterPro, and HMMER 3.0 was employed to search the jujube protein database with an E-value threshold of $<1 \times 10^{-5}$. Second, the BBX protein sequences of *Arabidopsis thaliana* (obtained from the PlantTFDB database) were used as queries in a BLASTP (in Tbttools 2.485) search against the jujube genome, with an E-value cutoff of $<1 \times 10^{-10}$. After removing redundant sequences, the candidate BBX protein sequences were submitted to the NCBI Conserved Domain Database (Batch CD-Search) for domain validation. Sequences with incomplete or missing conserved domains were discarded. Finally, the set of candidate BBX genes in jujube was determined by cross-referencing the results, as listed in Supplementary Table S1.

The physicochemical properties of ZjBBX proteins, including amino acid length, molecular weight (MW), and isoelectric point (pI), were analyzed using the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>, accessed on 3 April 2026). Subcellular localization was predicted using the Plant-mPLoC online server (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>, accessed on 3 April 2026) [25].

2.3. Phylogenetic Tree Construction of BBX Genes

The BBX protein sequences of *Arabidopsis thaliana* and *Malus domestica* were downloaded from the PlantTFDB database (<http://planttfdb.gao-lab.org/>, accessed on 3 April 2026) and combined with the jujube BBX protein sequences into a single FASTA file. Multiple sequence alignment of the amino acid sequences was performed using MEGA-X software 10.2.6. Based on the alignment results, a phylogenetic tree of BBX family members from the three species was reconstructed using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates. The resulting phylogenetic tree was visualized using the iTOL online tool (<https://itol.embl.de/>, accessed on 3 April 2026).

2.4. Analysis of Conserved Motif and Gene Structure

The exon-intron structures of the ZjBBX genes were determined using Tbttools 2.485 [26], based on the genome annotation data. Protein motif composition was analyzed using the MEME online tool (<http://meme-suite.org>, accessed on 3 April 2026) with

default parameters, searching for a maximum of 10 motifs, with widths ranging from 6 to 50 amino acids. The resulting data were visualized using TBtools [26].

2.5. Chromosomal Location and Duplication of ZjBBX Genes

Gene length and chromosomal start positions of the identified ZjBBX genes were extracted from the jujube genome annotation file and visualized using TBtools.

2.6. Analysis of Cis-Elements in the Promoter of ZjBBX Genes

The 2 kb upstream promoter sequences of the ZjBBX genes were extracted using TBtools and submitted to the PlantCare database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 3 April 2026) for cis-element prediction. The predicted cis-elements were then visualized with TBtools [26].

2.7. Transcriptome Analysis of ZjBBX Genes During Fruit Color and Abiotic Stress

Fruits of the jujube cultivar ‘Jinsi Xiaozao’ (JS) were collected at five developmental stages, designated F5 to F9. Each individual tree was considered one biological replicate, and fruits were randomly sampled from three trees per stage, yielding three biological replicates. For RNA extraction, eight fruits were harvested from each replicate, immediately cut into small pieces, rapidly frozen in liquid nitrogen, and stored at -80°C until use for transcriptome sequencing [21]. Moreover, at 1, 3, and 8 days post-treatment, phenotypic observations were recorded, and leaf samples were collected simultaneously, then immediately frozen in liquid nitrogen and stored at -80°C until use for transcriptome sequencing [22]. Total RNA was extracted from the aforementioned tissue samples and subjected to quality control checks. RNA integrity and concentration were assessed using an Agilent 2100 Bioanalyzer ($\text{RIN} \geq 7.0$) (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop 2000 spectrophotometer (A260/A280 ratio between 1.8 and 2.1) (Thermo NanoDrop 2000, ShangHai, China). After quality control, RNA-seq libraries were constructed using the NEBNext Ultra RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer’s instructions and sequenced on an Illumina HiSeq X Ten/NovaSeq 6000 platform (Illumina, San Diego, CA, USA) with a paired-end read length of 150 bp (2×150 bp). Raw reads were processed to obtain high-quality clean reads as follows. SeqPrep (v1.3.2, <https://github.com/jstjohn/SeqPrep>, accessed on 21 December 2022) and Sickel (v1.33, <https://github.com/najoshi/sickle>, accessed on 21 December 2022) were used to trim adapter sequences and remove low-quality bases, with default parameters. Reads with a Phred quality score below 20 over a sliding window of 5 bp were discarded, as were reads shorter than 50 bp after trimming. The clean reads were then aligned to the jujube reference genome (ASM3175591v1) using TopHat (v2.0.0, <http://tophat.cbcb.umd.edu/>, accessed on 21 December 2022) with default settings, allowing up to two mismatches per read. Gene-level read counts were quantified using the expectation-maximization algorithm implemented in RSEM (v1.3.1, <https://deweylab.github.io/RSEM/rsem-calculate-expression>, accessed on 21 December 2022), and transcript abundance was normalized to transcripts per million (TPM). All computational analyses were performed on the MajorBio cloud platform V1.0 (www.majorbio.com, accessed on 21 December 2022). Differential expression analysis was conducted using DESeq2 (v1.38.3) on the raw read counts, which were normalized internally by the median-of-ratios method to account for library size differences. Genes with an absolute $\log_2$ fold change ≥ 1 and an adjusted p -value (Benjamini–Hochberg corrected) < 0.05 were considered significantly differentially expressed. Gene Ontology (GO) enrichment was performed using Goatools (v1.3.0, <https://github.com/tanghaibao/GOatools>, accessed on 21 December 2022) with a hypergeometric test and a significance threshold of $p < 0.05$ after FDR correction. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

enrichment was analyzed using KOBAS (v3.0, <http://bioinfo.org/kobas>, accessed on 21 December 2022) with the same statistical cutoff. Protein–protein interaction networks were predicted using the STRING database (v11.5, <https://cn.string-db.org/>, accessed on 21 December 2022) with a confidence score threshold of ≥ 0.4 . Each experimental condition included three independent biological replicates, with each replicate subjected to independent library construction and sequencing to ensure statistical robustness. Gene expression heatmaps were generated using TBtools, and the results are presented in Supplementary Tables S2 and S3.

2.8. Transcriptome Analysis of ZjBBX Genes in Jujube Witches' Broom

Samples were collected from two jujube genotypes, the susceptible 'Fu' and the tolerant 'T13', at three time points after grafting: 1 month (S1), 1.5 months (S2), and 2 months (S3). For each genotype, scions were grafted onto two types of rootstocks: 'Fu' rootstocks infected with phytoplasma, and healthy 'Jiang' rootstocks (free of phytoplasma) which served as negative controls. This resulted in four grafting combinations per time point: Fu/Fu (infected), Fu/Jiang (healthy), T13/Fu (infected), and T13/Jiang (healthy), designated as Fu_F, Fu_J, T13_F, and T13_J, respectively. A total of 12 sample groups were collected, with three biological replicates per group (Supplementary Table S4-1). Phenotypic observations were conducted over a period of two years post-grafting, confirming that 'T13' exhibits tolerance and 'Fu' is susceptible to jujube witches' broom (JWB). Following phenotype recording, samples were rapidly frozen in liquid nitrogen and stored at -80°C for transcriptome sequencing [23]. Total RNA was extracted from the aforementioned tissue samples and subjected to quality control checks. After passing quality assessment, RNA-seq libraries were constructed and sequenced on an Illumina HiSeq X Ten/NovaSeq 6000 platform with a paired-end read length of 150 bp (2×150 bp). The raw reads were processed to obtain high-quality clean data. Specifically, SeqPrep (<https://github.com/jstjohn/SeqPrep>, accessed on 27 December 2022) and Sickle (<https://github.com/najoshi/sickle>, accessed on 27 December 2022) were used to trim adapter sequences and perform quality filtering, both with default parameters. After removing low-quality reads and adapters, the clean reads were aligned to the reference genome using TopHat v2.0.0 (<http://tophat.cbcb.umd.edu/>, accessed on 27 December 2022). Gene expression levels were quantified as transcripts per million (TPM) using RSEM (<https://deweylab.github.io/RSEM/rsem-calculate-expression>, accessed on 27 December 2022). All analyses were conducted on the MajorBio cloud platform. For differential expression analysis, the raw read counts were processed and normalized following standard pipelines. Differentially expressed genes (DEGs) between groups were identified using DESeq2, with significance thresholds of $|\log_2\text{FC}| \geq 1$ and adjusted p -value < 0.05 . Gene Ontology (GO) enrichment analysis was performed using Goatools v1.0 (<https://github.com/tanghaibao/GOatools>, accessed on 27 December 2022), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was analyzed using KOBAS (<http://bioinfo.org/kobas>, accessed on 27 December 2022). Protein–protein interaction networks were predicted using the STRING database (<https://cn.string-db.org/>, accessed on 27 December 2022). Each experimental condition included three independent biological replicates, and each replicate was subjected to sequencing to ensure statistical robustness. TBtools software was used to draw gene expression heatmap Supplementary Table S4-2.

2.9. Statistical Analysis

The experiment included three independent biological replicates and three technical replicates per sample. Data visualization was performed using Photoshop CC 2021.

3. Results

3.1. Identification and Characterization of ZjBBX Genes

In this study, a systematic analysis of the jujube genome identified a total of 26 *ZjBBX* genes (Table 1). The encoded proteins exhibited considerable variation in length, ranging from 105 amino acids (*ZjBBX18*) to 516 amino acids (*ZjBBX24*), with corresponding molecular weights of 12.37 kDa and 57.53 kDa. The isoelectric points (pI) ranged from 4.20 (*ZjBBX22*) to 9.31 (*ZjBBX23*), with 20 proteins classified as acidic (pI < 7) and 6 as basic (pI > 7: *ZjBBX3*, *ZjBBX7*, *ZjBBX18*, *ZjBBX23*, *ZjBBX25*, *ZjBBX26*). All *ZjBBX* proteins displayed negative GRAVY values, indicating hydrophilic properties. The instability index ranged from 37.90 (*ZjBBX1*) to 77.43 (*ZjBBX25*), with all but *ZjBBX1* exceeding 40, suggesting that the majority are unstable proteins. The aliphatic index ranged from 42.87 (*ZjBBX20*) to 79.93 (*ZjBBX23*), reflecting variation in predicted thermal stability. Subcellular localization prediction indicated that all 26 *ZjBBX* proteins are targeted to the nucleus, implying a potential regulatory role within the nucleus.

Table 1. Physical and chemical characteristics of *ZjBBX* genes in Chinese jujube.

Gene ID	Gene ID	Amino Acids (aa)	Theoretical pI	Molecular Weight (Da)	Instability Index	Aliphatic Index	Grand Average of Hydropathicity (GRAVY)	Subcellular Localization
<i>ZjBBX1</i>	LOC107419941	339.00	5.81	37,388.86	37.90	68.17	−0.445	Nucleus
<i>ZjBBX2</i>	LOC107433274	238.00	4.69	26,236.48	47.85	75.92	−0.406	Nucleus
<i>ZjBBX3</i>	LOC107405637	214.00	7.91	23,011.31	44.32	77.52	−0.137	Nucleus
<i>ZjBBX4</i>	LOC107426654	200.00	6.23	22,154.73	64.27	63.45	−0.418	Nucleus
<i>ZjBBX5</i>	LOC107418912	376.00	5.01	41,854.67	45.49	75.80	−0.499	Nucleus
<i>ZjBBX6</i>	LOC107432609	296.00	5.12	32,127.11	54.83	65.54	−0.363	Nucleus
<i>ZjBBX7</i>	LOC107424379	233.00	7.07	25,922.37	54.90	66.61	−0.521	Nucleus
<i>ZjBBX8</i>	LOC107415202	369.00	5.94	39,689.28	47.68	65.91	−0.302	Nucleus
<i>ZjBBX9</i>	LOC107426593	210.00	6.05	23,198.20	53.52	76.57	−0.483	Nucleus
<i>ZjBBX10</i>	LOC107420377	302.00	5.61	33,075.90	52.70	66.89	−0.430	Nucleus
<i>ZjBBX11</i>	LOC107405905	388.00	5.51	42,480.98	44.05	59.43	−0.621	Nucleus
<i>ZjBBX12</i>	LOC107435241	512.00	6.11	55,934.63	46.86	60.59	−0.627	Nucleus
<i>ZjBBX13</i>	LOC107426060	399.00	5.32	45,092.98	55.51	62.33	−0.757	Nucleus
<i>ZjBBX14</i>	LOC107424284	210.00	4.76	22,981.65	53.35	66.00	−0.352	Nucleus
<i>ZjBBX15</i>	LOC125422537	443.00	5.49	50,337.14	51.18	60.36	−0.820	Nucleus
<i>ZjBBX16</i>	LOC107429443	469.00	5.47	51,902.51	48.98	60.92	−0.851	Nucleus
<i>ZjBBX17</i>	LOC107416839	412.00	4.95	44,702.74	56.47	57.60	−0.502	Nucleus
<i>ZjBBX18</i>	LOC132803387	105.00	8.51	12,371.10	47.17	70.48	−0.604	Nucleus
<i>ZjBBX19</i>	LOC107430863	262.00	4.34	28,571.88	62.07	59.04	−0.810	Nucleus
<i>ZjBBX20</i>	LOC107422888	216.00	4.24	24,047.35	50.51	42.87	−1.284	Nucleus
<i>ZjBBX21</i>	LOC107417063	427.00	5.37	46,722.29	53.54	60.09	−0.496	Nucleus
<i>ZjBBX22</i>	LOC107416946	286.00	4.20	30,847.79	53.39	54.55	−0.973	Nucleus
<i>ZjBBX23</i>	LOC107429528	289.00	9.31	31,631.46	57.26	79.93	−0.098	Nucleus
<i>ZjBBX24</i>	LOC107413346	516.00	6.29	57,532.36	69.74	68.59	−0.599	Nucleus
<i>ZjBBX25</i>	LOC107432022	205.00	8.99	23,159.39	77.43	72.24	−0.491	Nucleus
<i>ZjBBX26</i>	LOC107410558	217.00	8.34	24,633.68	76.54	66.91	−0.704	Nucleus

3.2. Phylogenetic Analysis of the BBX Gene Family in Chinese Jujube

To investigate the phylogenetic relationships of BBX proteins in jujube, a phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA X, incorporating BBX protein sequences from jujube (26), *Arabidopsis thaliana* (32), and apple (60). As shown in Figure 1, the *ZjBBX* proteins were classified into five major groups (I–V). Group IV contained the fewest members (7.69%, 2/26), while Group I comprised the largest proportion (38.46%, 10/26), suggesting that this group may have undergone multiple gene duplication events. Notably, most jujube BBX genes clustered closely with apple homologs at the terminal branches of the phylogenetic tree, indicating a relatively close evolutionary relationship between the two species.

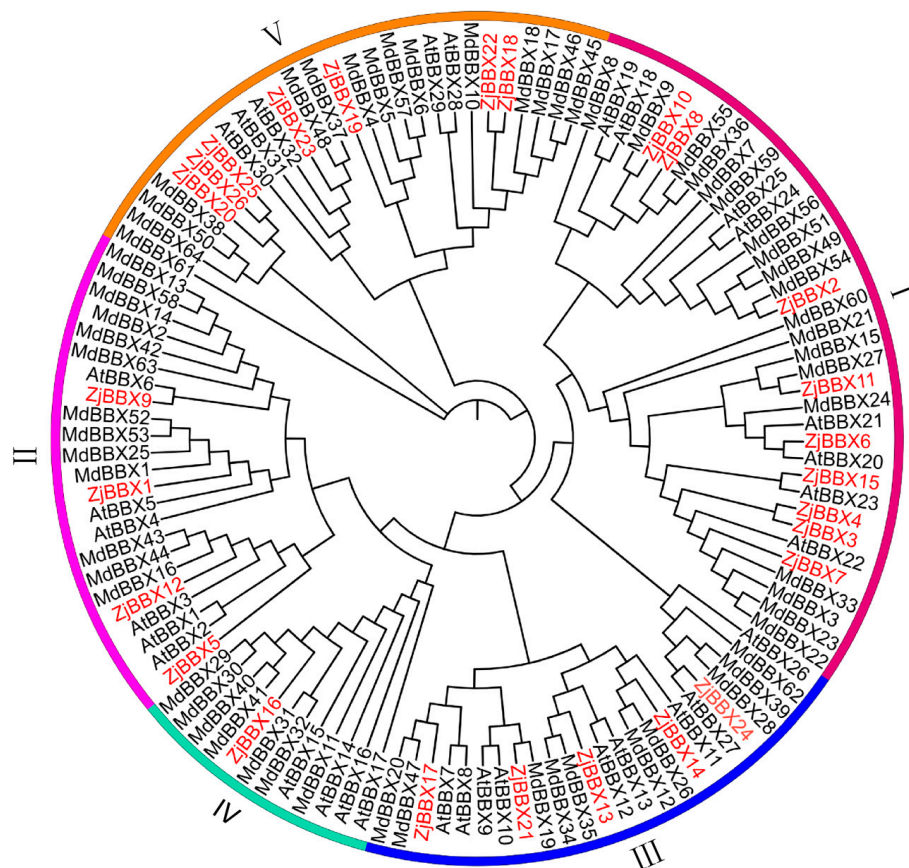


Figure 1. A neighbor-joining (NJ) phylogenetic tree was constructed based on the amino acid sequences of BBX proteins from *Ziziphus jujuba* (ZjBBX), *Arabidopsis thaliana* (AtBBX), and *Malus domestica* (MdBBX), with 1000 bootstrap replicates. The resulting tree classified all BBX members into five distinct groups, each distinguished by a different color. ZjBBX genes are highlighted in red for clarity. Note: A neighbor-joining (NJ) phylogenetic tree was constructed based on the amino acid sequences of BBX proteins from *Ziziphus jujuba* (ZjBBX), *Arabidopsis thaliana* (AtBBX), and *Malus domestica* (MdBBX), with 1000 bootstrap replicates. The resulting tree classified all BBX members into five distinct groups (I–V), each distinguished by a different color. ZjBBX genes are highlighted in red for clarity.

3.3. Structural Analysis and Motif Composition of the BBX Gene Family in Chinese Jujube

To investigate the structural features of ZjBBX genes, we analyzed their genomic DNA sequences in relation to the corresponding protein sequences. Ten conserved motifs were identified in the ZjBBX proteins using the MEME online tool (Figure 2A). The number of motifs per protein ranged from 2 to 5, and the motif distribution was largely conserved among members within the same subfamily. Based on domain composition, the ZjBBX members were categorized into four types: those containing one B-box (7 genes), two B-boxes (8 genes), one B-box plus one CCT domain (2 genes), and two B-boxes plus one CCT domain (9 genes) (Figure 2B).

Exon–intron structures were determined based on the genomic sequences of each ZjBBX gene (Figure 2C). Structural variations were observed among different phylogenetic branches. Notably, most ZjBBX genes contained introns, with the exception of ZjBBX23, which lacked introns entirely. The presence of introns generally suggests transcriptional diversity and functional complexity. Whether the observed intron patterns in ZjBBX genes are evolutionarily inherited or acquired through mutation over time, and how they may affect gene function, warrants further investigation.



Figure 2. Conserved motifs and structural features of *ZjBBX* genes. **(A)** The ten conserved motifs (motifs 1–10) identified in *ZjBBX* proteins are shown in distinct colors. **(B)** Domain compositions of *ZjBBX* family members. **(C)** Gene structures of *ZjBBX* genes, with coding sequences (CDS) and introns represented by blue boxes and black lines, respectively.

3.4. Chromosome Localization and Duplication Analysis of the *ZjBBX* Genes

To determine the chromosomal distribution of the *ZjBBX* genes, a localization analysis was performed. The results showed that the 26 *ZjBBX* genes were unevenly distributed across 10 chromosomes (Figure 3), with numbers per chromosome ranging from one to six. Specifically, chromosome 4 harbored the most (6 genes), followed by chromosome 12 (4 genes), chromosomes 1 and 9 (3 genes each), chromosomes 2, 5, 7, and 11 (2 genes each), and chromosomes 3 and 10 (1 gene each).

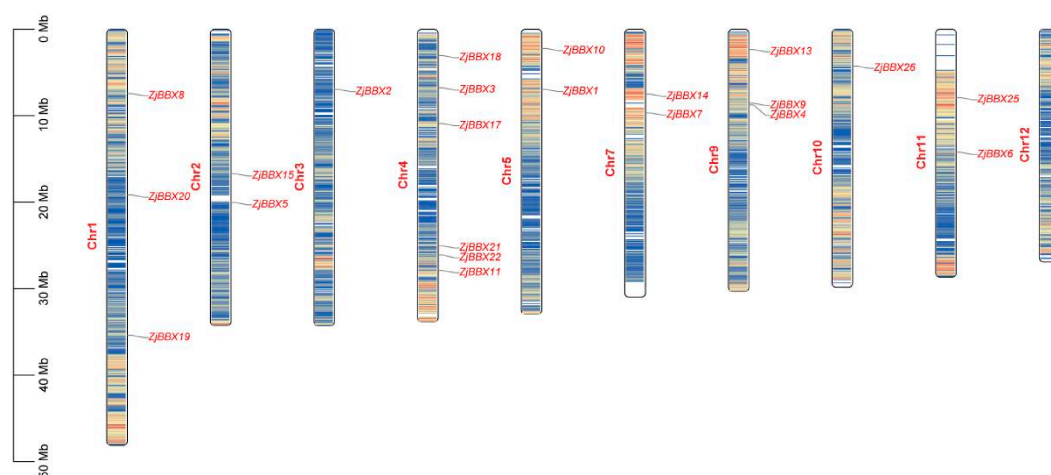


Figure 3. Chromosomal distribution of the BBX genes across the ten chromosomes of jujube. Chromosomes are represented by colored rectangles, and the scale bar on the left indicates their lengths in megabases (Mb).

To elucidate the amplification mechanism of the BBX gene family in jujube, intraspecific collinearity analysis was performed using MCScanX in TBtools 2.485. The results

indicated that segmental duplication events were the primary driver of *ZjBBX* gene amplification, whereas no tandem duplication events were detected (Figure 4). A total of nine segmental duplication events were identified on chromosomes 1, 2, 4, 5, 7, 9, 10, 11, and 12, generating eight pairs of duplicated genes, including *ZjBBX4/ZjBBX10*, *ZjBBX5/ZjBBX7*, *ZjBBX7/ZjBBX9*, *ZjBBX7/ZjBBX10*, *LOC125422538/ZjBBX16*, *ZjBBX19/ZjBBX20*, *ZjBBX19/ZjBBX22*, and *ZjBBX25/ZjBBX26*. These findings suggest that segmental duplication plays a significant role in the expansion and evolutionary diversification of the *ZjBBX* gene family.

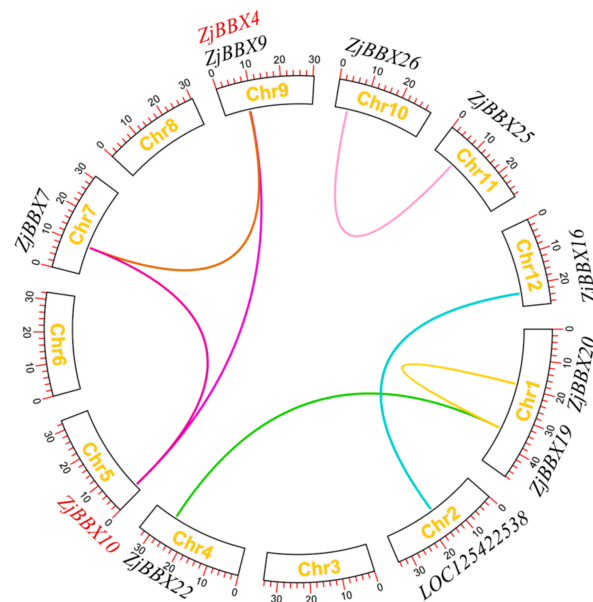


Figure 4. Intraspecific collinearity of BBX genes in jujube. Purple, blue, and green lines represent segmental duplicated gene pairs among different chromosomes.

3.5. Promoter Cis-Acting Elements Analysis of the *ZjBBX* Genes

To elucidate the potential regulatory mechanisms of *ZjBBX* genes, the 2000 bp promoter regions upstream of the start codons were analyzed using the PlantCARE database. A total of 2751 cis-acting elements (CREs) were identified and classified into four major categories. The majority were core promoter elements (2029, 73.75%), followed by elements involved in growth and development (468, 17.01%), hormone response elements (223, 8.11%), and stress response elements (31, 1.13%) (Figure 5A). All 26 *ZjBBX* genes contained core promoter, hormone response, and growth/development elements, while 19 genes also harbored stress-related CREs (Figure 5B,C).

Among the core promoter elements, the most abundant were TATA-box (1586, 57.65%) and CAAT-box (432, 15.70%). For hormone-responsive CREs, the predominant motifs included ABRE (90, 3.30%), CGTCA- and TGACG-motifs (35 each, 1.27%), and AuxRR-core (6, 0.22%), with SARE being the least frequent (1, 0.036%). In the growth- and development-related category, G-Box (103, 3.74%) and Box 4 (82, 2.98%) were the most common. Among stress-responsive elements, MBS (20, 0.73%), TC-rich repeats (11, 0.40%), and LTR (10, 0.36%) were detected. Notably, promoter regions of *ZjBBX22* and *ZjBBX19* contained the highest numbers of CREs (221 and 172, respectively), while *ZjBBX7* and *ZjBBX17* had the fewest (61 each) (Figure 5). The diverse array of cis-elements identified in the *ZjBBX* promoters suggests potential involvement of these genes in growth, development, and stress responses, warranting further functional investigation.

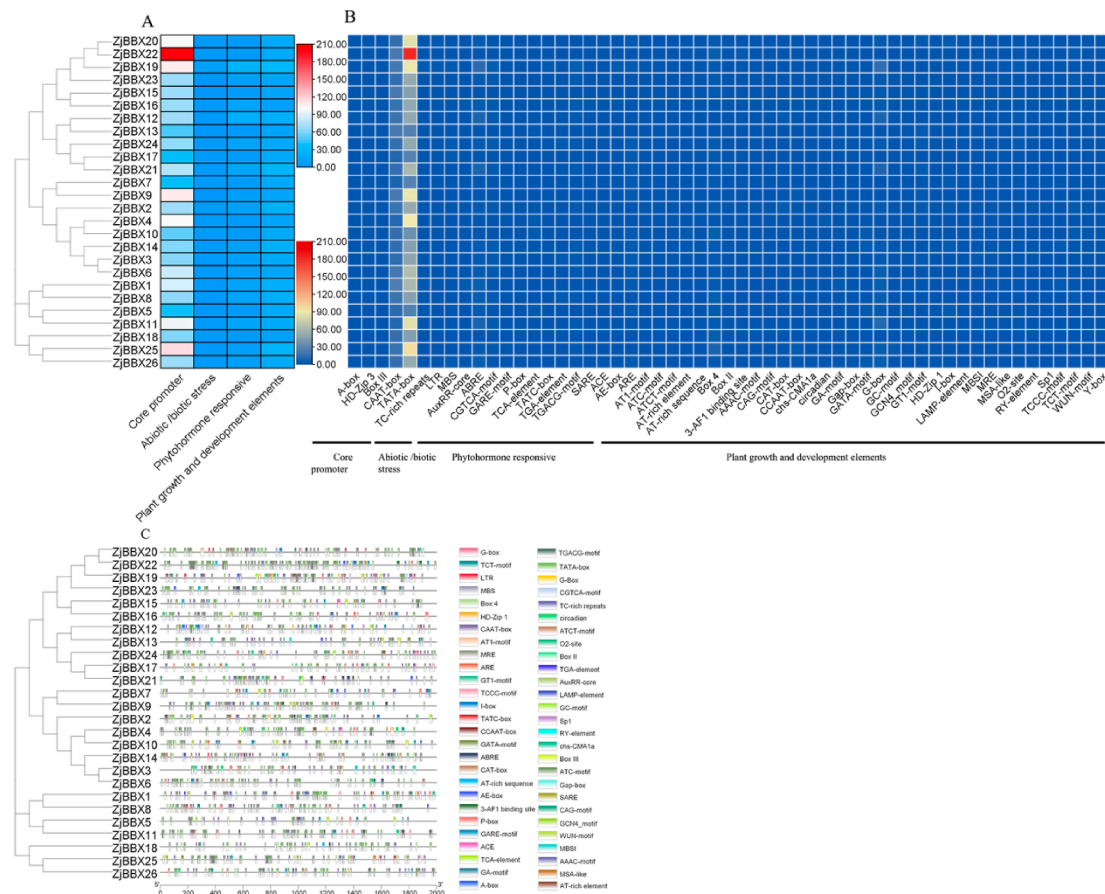


Figure 5. Prediction and visualization of cis-acting elements in the promoters of *ZjBBX* genes. (A) Number of cis-acting elements detected in each *ZjBBX* promoter, categorized into four functional types. (B) Proportional distribution of the four categories: core promoter elements, stress-responsive elements, phytohormone-responsive elements, and growth/development-related elements. (C) Physical distribution of cis-acting elements along the upstream promoter sequences of *ZjBBX* genes, visualized using TBtools. The x-axis represents the promoter region upstream of the transcriptional start site (TSS). Colored boxes indicate different types of cis-elements, with their horizontal positions and widths corresponding to the exact start sites and physical lengths on the genomic sequence.

3.6. Expression Profiles of ZjBBX Genes in Fruit Coloring and Salt Stress Responses

To explore the potential involvement of *ZjBBX* genes in fruit coloration, their expression profiles were analyzed using published transcriptomic data (Figure 6). Among the 26 *ZjBBX* genes, nine exhibited marked expression changes during fruit coloring. Seven genes (*ZjBBX1*, *ZjBBX2*, *ZjBBX10*, *ZjBBX13*, *ZjBBX15*, *ZjBBX16*, and *ZjBBX17*) showed progressive down-regulation, whereas *ZjBBX9* and *ZjBBX25* displayed sustained up-regulation (Figure 6A), suggesting their possible participation in fruit coloration.

Under salt stress, expression patterns of *ZjBBX* genes also varied considerably. Transcript levels of *ZjBBX1*, *ZjBBX2*, *ZjBBX8*, and *ZjBBX11* declined progressively with prolonged Na^+ treatment. In contrast, *ZjBBX17*, *ZjBBX13*, and *ZjBBX24* were rapidly up-regulated within 6 h of treatment, while *ZjBBX7*, *ZjBBX9*, *ZjBBX22*, and *ZjBBX23* exhibited sharp decreases (Figure 6B). These results suggest that different *ZjBBX* members may play distinct regulatory roles in salt stress responses. Genes showing sustained downregulation (e.g., *ZjBBX1*, *ZjBBX2*, *ZjBBX8*, and *ZjBBX11*) may be involved in adaptive regulatory processes, whereas those with rapid early up-regulation (*ZjBBX17*, *ZjBBX13*, and *ZjBBX24*) could function as early responsive components. Conversely, genes that were sharply down-

regulated (*ZjBBX7*, *ZjBBX9*, *ZjBBX22*, and *ZjBBX23*) might indirectly contribute to salt tolerance by modulating downstream signaling pathways.

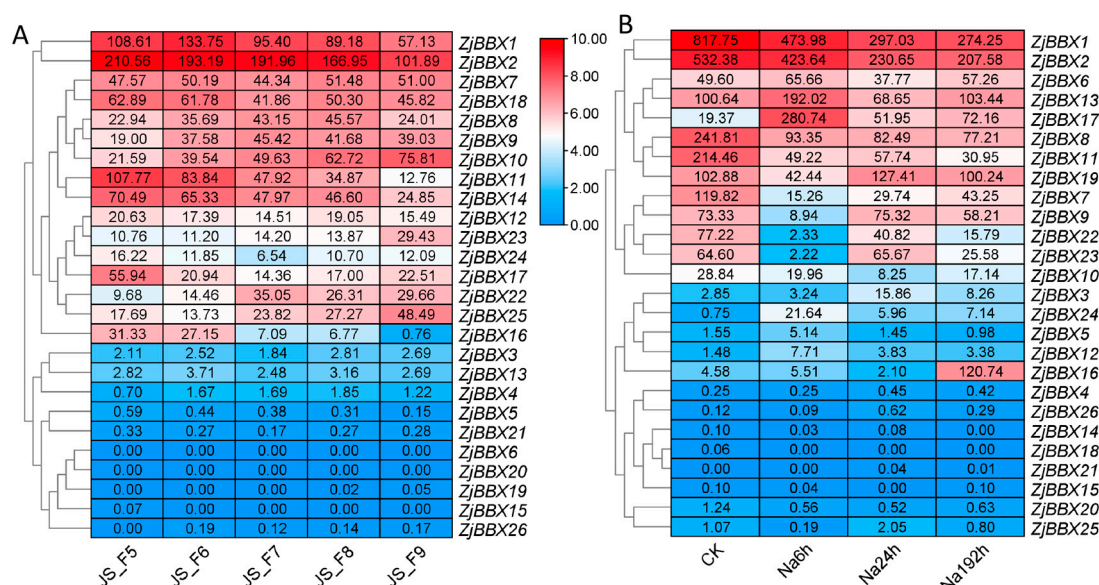


Figure 6. Expression profiles of *ZjBBX* genes based on RNA-seq data. (A) Expression patterns during fruit coloration. (B) Expression patterns under salt stress. Gene expression levels are presented as FPKM (Fragments Per Kilobase of transcript per Million mapped reads). The heatmaps display FPKM; red indicates up-regulation, blue indicates down-regulation, and color intensity reflects the magnitude of transcriptional change.

3.7. Expression Analyses of *ZjBBX* Genes During Jujube Witches' Broom

To investigate the potential involvement of *ZjBBX* genes in biotic stress responses, their expression profiles were analyzed using RNA-seq data from healthy and jujube witches' broom (JWB)-infected trees (Figure 7). Among the 26 *ZjBBX* genes, 13 exhibited notable expression changes. Nine genes (*ZjBBX1*, *ZjBBX2*, *ZjBBX6*, *ZjBBX7*, *ZjBBX9*, *ZjBBX15*, *ZjBBX17*, *ZjBBX19*, and *ZjBBX23*) were highly expressed in both healthy and infected materials, suggesting that they may be primarily involved in growth and development rather than specifically associated with JWB response.

In contrast, *ZjBBX8*, *ZjBBX11*, and *ZjBBX13* displayed distinct expression patterns between healthy and diseased samples. *ZjBBX8* showed relatively stable expression across the three time points in healthy plants, but exhibited a sustained increase in infected materials. *ZjBBX11* showed similar expression trends between healthy and diseased plants in the first two stages, but diverged in the third stage, with higher transcript levels in infected tissues, which may correlate with the flower-to-leaf malformations characteristic of JWB infection. Meanwhile, *ZjBBX13* exhibited stable expression in diseased plants but increased progressively in healthy ones, implying a potential role in regulatory processes under normal conditions. Notably, *ZjBBX22*, which clusters in the same phylogenetic branch as *ZjBBX8*, *ZjBBX11*, and *ZjBBX13*, did not show differential expression, suggesting it may not be involved in JWB response.

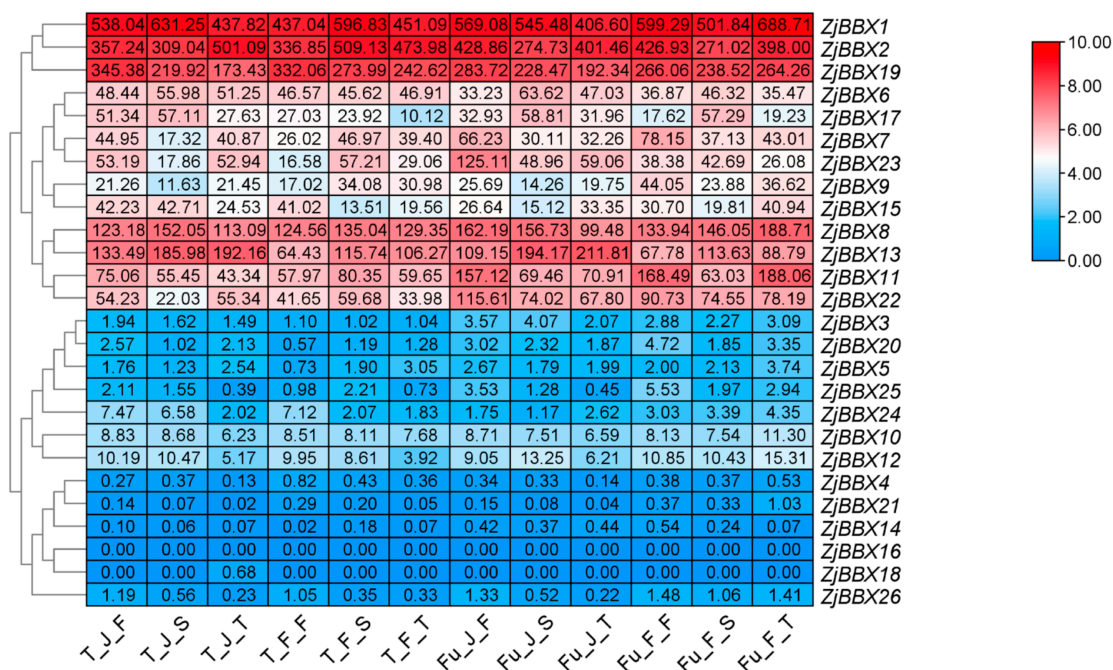


Figure 7. Expression patterns of *ZjBBX* genes under jujube witches’ broom (JWB). The heatmap displays differential gene expression between infected (diseased) and healthy samples. Transcript abundances were quantified as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and subsequently transformed to FPKM for heatmap visualization. The color scale (shown in the legend) represents FPKM, with red indicating up-regulation and blue indicating down-regulation.

4. Discussion

BBX proteins constitute a family of transcription factors involved in plant growth, development, and responses to abiotic and biotic stresses [11]. To date, genome-wide identification of BBX family members has been performed in various dicot and monocot species, including 32 members in *Arabidopsis thaliana* [27], 30 in rice [28], 29 in tomato (*Solanum lycopersicum*) [29], 30 in potato (*Solanum tuberosum*) [30], and 37 in pear (*Pyrus bretschneideri*) [31]. However, no such identification or expression analysis has been reported in jujube. In this study, we systematically identified and characterized the BBX gene family in jujube using the T2T genome assembly, obtaining a total of 26 *ZjBBX* genes. This number differs somewhat from those reported in other plant species, which may be attributed to variations in genome size, evolutionary history, and the frequency of whole-genome duplication (WGD) or segmental duplication events among species. Consistent with previous studies [27,32,33], the jujube BBX proteins were classified into five subfamilies (I–V) based on sequence similarity with BBX proteins from *Arabidopsis* and apple. This classification pattern is largely congruent with that reported in other species, suggesting that the BBX gene family in jujube has been evolutionarily conserved.

Gene structural diversity often plays an important role in the evolution of multigene families. BBX family proteins typically contain one or two conserved B-box domains, and some members also possess CCT domains [27]. Similar structural features were observed in the 26 *ZjBBX* proteins identified in jujube, suggesting that BBX proteins are relatively conserved across species. Within the same phylogenetic group, *ZjBBX* genes shared similar intron/exon arrangements and motif compositions, implying that they may perform analogous biological functions. Based on domain composition, the 26 *ZjBBX* genes were classified into four types: those containing one B-box (7 genes), two B-boxes (8 genes), one B-box plus one CCT domain (2 genes), and two B-boxes plus one CCT domain (9 genes). Comparable distribution patterns have been reported in other dicots, with corresponding

numbers of 7, 8, 4, and 13 in *Arabidopsis* [27]; 6, 9, 4, and 7 in pear [31]; and 6, 10, 5, and 8 in tomato [30]. In contrast, monocots exhibited markedly different distributions: 2, 8, 9, and 5 in sorghum [34]; 4, 17, 10, and 5 in maize [35]; and 3, 10, 10, and 7 in rice [28]. These differences suggest that independent amplification events occurred after the divergence of monocots and dicots [31,33]. Gene duplication events are recognized as major drivers of new gene generation and genome evolution [36]. In jujube, a total of nine segmental duplication events were identified within the *ZjBBX* gene family, producing eight pairs of duplicated genes, indicating that segmental duplication has contributed substantially to the expansion of this family. The relatively limited number of such duplication events may also account for the moderate size of the *ZjBBX* family in jujube.

Cis-regulatory elements in promoter regions are equally important for functional diversification [37], as they mediate transcriptional regulation through specific transcription factor binding [38]. In this study, a large number of cis-elements were predicted in the *ZjBBX* promoters, implicating these genes in diverse processes including growth, development, phytohormone responses, and abiotic and biotic stress responses [27,39–41]. These findings suggest that the BBX family in jujube may participate in multiple physiological and stress-related pathways, although these inferences remain preliminary and require experimental validation (e.g., EMSA, ChIP, and promoter–reporter assays). Transcriptomic analyses have further indicated that *ZjBBX* genes may be involved in fruit coloration, salt stress responses, and disease defense in jujube. Although BBX genes have been reported to regulate fruit coloration in pear, apple, strawberry, and cherry [42–47], no similar study has been conducted in jujube. Regarding salt stress, studies in *Arabidopsis*, wheat, tomato, ginkgo, and apple have demonstrated that BBX genes enhance salt tolerance [48–52]; however, their specific roles in jujube remain to be elucidated. Jujube witches’ broom (JWB) is a devastating phytoplasma-borne disease with no effective cure. Our findings suggest that *ZjBBX* genes may contribute to defense against JWB, as their expression patterns correlate with disease progression. Similar putative functions in biotic stress responses have been reported for BBX homologs in sweet potato, pepper, and sugar beet [14–16].

Collectively, this comprehensive analysis of the *ZjBBX* gene family in jujube not only details the sequence and structural characteristics of these genes but also provides new insights into their potential regulatory roles in fruit coloration, salt tolerance, and disease resistance.

5. Conclusions

This study presents the first systematic characterization of the BBX gene family in jujube, integrating phylogenomic, structural, and multi-stress expression analyses. Our findings establish three key lines of evidence linking *ZjBBX* genes to agronomically important traits, persistent up-regulation of *ZjBBX9/25* during fruit coloration, divergent salt-response patterns among family members, and disease-associated expression of *ZjBBX8/11/13* under witches’ broom infection. Beyond providing a foundational platform for functional validation, our work uncovers novel candidate regulators that distinguish jujube BBXs from their counterparts in other species in terms of stress response diversity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070816/s1>, Table S1. Jujube BBX protein sequences; Table S2. Fruit color transcriptome data; Table S3. Transcriptome data of sour jujube seedlings under salt stress treatment; Table S4-1. Experimental design for phytoplasma inoculation, sample labeling, and transcriptome sampling; Table S4-2. Transcriptome data of tolerant and susceptible materials under jujube witches’ broom stress.

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