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Genome-Wide Identification of the Sweet Orange *bZIP* Gene Family and Analysis of Their Expression in Response to Infection by *Penicillium digitatum*

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Abstract: (1) Background: The sweet orange (*Citrus sinensis*) is the most widely cultivated and productive citrus fruit in the world, with considerable economic value and good prospects for development. However, post-harvest storage and transport of the fruit are often affected by infestation by *Penicillium* species, leading to many losses. (2) Methods: In this study, the family of *bZIP* genes from the whole genome of sweet orange was identified and analyzed in detail in terms of gene structure, physicochemical properties, protein structure, conserved structural domains, chromosomal positioning, and promoter analysis using bioinformatic analysis, in addition to an analysis of the expression patterns of the fruit following *Penicillium* infection. (3) Results: In this study, 50 *CsbZIP* genes were identified from the sweet orange genome. In silico analysis showed that *Cs_ont_3g005140* was presumably localized in the chloroplasts, while the rest of the family members were located in the nucleus. Phylogenetic trees of grape, apple, *Arabidopsis*, and sweet orange were constructed on the basis of evolutionary relationships and divided into 16 subfamilies. Conserved motif analysis showed that all *CsbZIP* family genes encode proteins containing the highly conserved Motif 1. Promoter prediction analysis showed the chromosomal positioning, and the covariance analysis showed that the 50 *CsbZIPs* were unevenly distributed on nine chromosomes, with 10 pairs of duplicated genes. In the analysis of expression patterns, 11 of the 50 *CsbZIP* genes were not expressed, 12 were upregulated, 27 were downregulated, and five of the upregulated genes were highly expressed. (4) Conclusions: In this study, two *CsbZIP* members were each closely related to two *Arabidopsis thaliana* genes associated with salt stress. The functions of the replicated and re-differentiated *CsbZIP* homologs (*Cs_ont_1g027160* and *Cs_ont_8g020880*) divergee further, with one responding to inoculation by *Penicillium* and the other not doing so. Five genes associated with sweet orange in response to *Penicillium* infestation were initially screened (*Cs_ont_3g000400*, *Cs_ont_3g003210*, *Cs_ont_5g007090*, *Cs_ont_5g011180*, *Cs_ont_8g020880*). This study provides some theoretical basis for subsequent research into the response mechanism of sweet orange *bZIP* transcription factors under biotic stresses.

Keywords: sweet orange; *bZIP* transcription factor; *Penicillium*; biological stress; stress resistance; response mechanisms

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1. Introduction

Citrus sinensis, a genus of citrus in the *Rutaceae* family, is the world's largest cultivated and highest-yielding fruit [1]. Statistics show that in 2017, the global citrus cultivation area was 143 million mu, with production reaching 184 million tons. The annual output of sweet

oranges worldwide accounts for more than half of the total citrus production, with very significant production and economic value. The main production areas of sweet oranges in the world are concentrated in China, Brazil, and the United States, and the planting areas in China are located in Sichuan, Hubei, Hunan, Guangdong, and Yunnan. The ripening period of sweet oranges covers almost the whole year, and it has good economic value and development prospects [2]. Citrus fruits are generally used for fresh eating. In citrus, *Penicillium italicum* is a prime disease during post-harvest storage and transportation [3], with more than 70% caused by *Penicillium italicum* and *P. digitatum* [4].

Transcription factors (TFs) are a group of proteins that regulate gene expression by binding specifically to downstream gene promoter cis-elements or interacting with other proteins [5]. Basic leucine zipper (*bZIP*) [6] transcription factors are commonly found in eukaryotic genomes and are among the relatively large and conserved gene families identified so far [7], with vital regulatory functions in plants' growth and development and stress responses [8]. Studies have shown that members of the *bZIP* gene family can alter their specificity and affinity for DNA binding through dimerization, phosphorylation modifications, or interactions with other proteins, thereby affecting the stability of the target genes and themselves, and thus participating in the regulation of physiological and biochemical processes in plants and enhancing their stress resistance [9].

The *bZIP* transcription factor gene family has been identified in several plant genomes, such as *Arabidopsis thaliana* [10], *Armeniaca mume* [11], *Hevea brasiliensis* [12], *Populus tremula* [13], *Vitis vinifera* [14], and *Actinidia chinenssis* [15]. Several studies have shown that *bZIP* transcription factors play a vital regulatory role in the abiotic stresses of plants. Guo et al. (2020) found the *BpbZIP1* gene from *Betula platyphylla*, showing that the gene can respond to salt stress and drought stress, and that the gene expression product of *BpbZIP1* could improve salt tolerance in *Betula platyphylla* by scavenging reactive oxygen species [16]. Overexpression of the Subclade A transcription factor *ZmbZIP72* in *Zea mays* under drought or salt stress exhibited greater tolerance to adversity [17]. Expression of the *CsbZIP6* gene of *Camellia japonica* is induced under low-temperature conditions, and this gene plays a negative regulatory role in low-temperature stress [18]. Himanshu Tak et al. showed that the expression of *VvbZIP23* was induced by a wide range of abiotic stresses, including drought, salt, and cold. Exogenous signaling chemicals such as abscisic acid, methyl viologen, salicylic acid, jasmonic acid, and vinblastine also induced the expression of *VvbZIP23* [19]. The results of Li et al. confirmed the vital role of *CsBZIP40* in improving resistance to citrus rot through the salicylic acid (SA) signaling pathway, and that the presence of *NPR1* activates the PR gene [20].

Much progress in research into *bZIP* transcription factors has been made in the past. However, most of these advances are related to the involvement of these proteins in abiotic stress, and in seed and plant development, and there are few examples of research on biotic stress. There are only a few studies on sweet orange's *bZIP* transcription factors related to biotic stresses, so it is vital to study the changes in the *bZIP* transcription factors in response to sweet orange's disease susceptibility to develop new varieties of sweet orange or citrus resistant to diseases. In this study, bioinformatic analysis was used to identify the *bZIP* gene family from the sweet orange genome and to analyze its physicochemical properties, phylogenetic relationships, gene structure, promoter elements, gene localization, and expression patterns following *Penicillium* infestation. Sweet oranges collected from Chu Orange Manor in Xinping, Yuxi, Yunnan Province, China, were selected as the experimental material. The transcriptomic data of disease-infected fruits were obtained by inoculation via acupuncture with *Penicillium* [21]. Combined with the bioinformatic analysis methods, the differences in the expression of the *bZIP* transcription factors between disease-infected fruits and the control groups were studied to mine the candidate genes for disease resistance in sweet oranges, to provide a theoretical basis for exploring the molecular mechanism of disease resistance in sweet oranges and cultivating new varieties of sweet oranges with disease resistance.

2. Materials and Methods

2.1. Identification of the bZIP Gene Family in Sweet Orange

Download the sweet orange whole genome protein sequence, the genome sequence, the CDS sequence, and annotation files [22] from the CPBD(Citrus Pan-genome to Breeding Database) [23](http://citrus.hzau.edu.cn/, accessed on 24 June 2022). The protein sequences encoded by the *A. thaliana bZIP* genes were downloaded from the TAIR [24] (http://www.arabidopsis.org/index.jsp, accessed on 24 June 2022) database. The Hidden Markov Model (PF00170) analysis was downloaded from the Pfam database [25] (http://pfam.sanger.ac.uk/, accessed on 5 July 2022). Two methods were used to identify the *bZIP* gene members from the sweet orange genome. First, Blastp of Blast+ software was used to blast sweet orange proteins with proteins of *A. thaliana bZIP* genes as a reference, and proteins that showed high homology (E-value < 1×10^{-5}) with protein sequences of *A. thaliana bZIP* genes were filtered out from sweet orange. Then, the hmmsearch program of HMMER software was used to search protein sequences containing the domain of *bZIP* genes (ID: PF00170) from blast results, and all protein sequences with an E-value lower than 1×10^{-5} were reserved. The other method is to directly search the protein sequences of all sweet orange genes for the *bZIP* conserved structural domain (PF00170) and finally retain the sweet orange protein sequences containing the conserved structural domain of the *bZIP* gene. After the results of both identifications were combined, the *bZIP* gene family members were obtained. To ensure all *bZIP* gene family members contained complete structural domains, we used the online website HMMER [26] (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan, accessed on 8 July 2022) to remove sequences that did not have conserved structural domains or had incomplete structural ones. The preserved sequences are the members of the sweet orange *bZIP* gene family identified in this study and will be used for further bioinformatics analysis.

2.2. Physicochemical Properties, Predicted Secondary Structure, and Subcellular Localization of the Sweet Orange bZIP Gene

An analysis of the physicochemical properties is vital for analyzing the structure and function of proteins [6]. In this study, physicochemical properties such as the amino acid number, molecular weight, theoretical isoelectric point, instability coefficient, lipid coefficient, and hydrophilicity of the proteins encoded by the sweet orange *bZIP* gene family obtained from the identification were predicted using the online tool Expasy (https://web.expasy.org/protparam/, accessed on 16 July 2022). The subcellular localization of the sweet orange *bZIP* genes was predicted and analyzed similarly by entering the amino acid sequences of the target genes to the Plant-mPLoc website (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 17 July 2022). The website SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa, accessed on 18 July 2022) was used to analyze the secondary structure of the sweet orange *bZIP* protein and obtain the proportion of α-helices, β-folds, extended chains, and irregular coils. The values of parameters such as the physicochemical properties, secondary protein structures, and subcellular localization of sweet orange *bZIP* transcription factor family members were summarized and recorded in an Excel sheet and later analyzed.

2.3. Phylogenetic Analysis of the Sweet Orange bZIP Gene Family

The phylogenetic analysis of the sweet orange *bZIP* gene family was carried out in four steps [27]. In the first step, the sequences of *Arabidopsis bZIP* protein were merged and downloaded from the TAIR [24] database, the GDR [28] (http://www.rosaceae.org/, accessed on 22 July 2022) database, and the GENESCOPE [14] (http://www.genescope.cns.fr, accessed on 23 July 2022) database to obtain the *bZIP* protein sequences of *Arabidopsis*, apple, and grape, and to identify the obtained sweet orange *bZIP* protein sequences, which were integrated and saved to an Excel sheet. In the second step, these sequences were multiply aligned by ClustalW (version 2.0) [29] software. The third step was to select the most suitable model and build the tree, combined with the sequence comparison results,

using IQ-TREE (version 1.6.12) [30] software to filter the optimal model for the *bZIP* family's phylogenetic tree. After selection of the optimal model, the bootstrap value was set to 1000, and aln files were obtained to build the evolutionary tree. The fourth step was visualization using the online software ChiPlot (https://www.chiplot.online/, accessed on 25 July 2022) for the evolutionary tree.

2.4. Analysis of the Conserved Motifs and Gene Structure of the Sweet Orange bZIP Gene Family

Proteins with the same or similar conserved motifs in plants perform the same functions [31]. In this study, the conserved motifs of the sweet orange *bZIP* gene were searched using the online tool MEME [32] (http://meme-suite.org/, accessed on 26 July 2022), with the maximum number of motifs searched set to 10, with other parameters set to the defaults. Finally, on the basis of the annotated files of the sweet orange genome and the conserved motif search results, the Gene Structure View tool in TBtools (version 1.0987) [33] was used to visualize and embellish the conserved motifs and gene structure of sweet orange *bZIP.*

2.5. Analysis of the Sweet Orange bZIP Gene Family's Cis-Acting Elements

The paralogs with different cis-acting elements tend to have different expression patterns [34]. Cis-acting elements include promoters, enhancers, regulatory sequences, and inducible elements that regulate gene expression. A cis-acting element's sequence does not encode any protein but merely provides a site of action to interact with the trans-acting element. In this study, after the genome annotation file of sweet orange had been obtained, the GXF Sequences Extract tool in TBtools [33] software was used to extract the 2000 bp gene sequence upstream of each sweet orange *bZIP* gene and upload it to the online promoter prediction tool PlantCARE [35] (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 27 July 2022) for predicting the promoter cis-acting elements. Finally, the results were collated, visualized, and embellished using the Gene Structure View tool in TBtools software.

2.6. Analysis of the Gene Localization and Covariance of the Sweet Orange bZIP Gene Family

Colinearity refers to the large-scale homologous gene pairs in order of chromosomes [36]. The genome and annotation files of sweet orange and *Arabidopsis* were first obtained, then the *bZIP* genes of sweet orange and *Arabidopsis* were compared and analyzed by the One Step MCScanX tool in TBtools [33] software. Finally, the data were uploaded to the online tool ChiPlot (https://www.chiplot.online/, accessed on 30 July 2022) to plot and map the collinearity and chromosome distribution of the sweet orange *bZIP* gene family.

2.7. Analysis of the Tissue Expression of the Sweet Orange bZIP Gene Family

Transcriptomic data under biotic stresses caused by *Penicillium* infestation were analyzed in this study. In the first step, *P. digitatum* was isolated and purified from diseased sweet orange spots using the streak plate method. In the second step, the inoculum was inoculated onto a potato medium using the plate coating method and incubated in a incubator at a constant temperature of 25 °C. In the third step, the spore suspension was injected onto a frozen orange pericarp as the treatment group, while an injection of an equal amount of autoclaved distilled water was used as the control. In the fourth step, the inoculated fruits were incubated in on ultra-clean bench at 25 °C for seven days, and the samples were stored in liquid nitrogen and sent to Bioyi Biotechnology Company for extraction of the total RNA and cDNA library construction, followed by sequencing using Illumina HiSeq to obtain the transcriptomic data of citrus under biotic stress. The resulting sweet orange RNA-Seq data were submitted to the NCBI repository SRA (accession numbers SRR19976158, SRR19976159, SRR19976157, SRR19976160, SRR19976161, and SRR19976156) [37].

To avoid many adapters and low-quality data in the transcriptomic data, we used Trim-galore and Trimmomatic software to remove the adapters and filter out low-quality data. FastaQC (version 0.11.9) was used to check the quality of the data, finally ensuring

that 90% of the transcriptomic data had a Q-value > 30. Reads from transcripts that passed the quality control step were mapped to the reference genome (*C. sinensis* genome v3.0) by hisat2, and the FeatureCounts toolkit of Rsubread software (version 2.12.2) was used to count the reads compared with those to the reference genome. To calculate the FPKM (fragments per kilobase of the exon model per million mapped fragments) for each gene, the expression of each gene was quantified. A heat map of the expression patterns of the sweet orange *bZIP* gene was constructed using the R package Pheatmap on the basis of the $\log_2$ (FPKM + 1) values. Furthermore, the differential expression levels of sweet orange genes under biological stress were analyzed via the method of Wei et al. [38] and Zhang et al. [39].

3. Results and Analysis

3.1. Identification and Characterization of the bZIP Transcription Factor Families of Sweet Orange

In total, 112 and 125 sweet orange *bZIP* genes were retrieved by HMMer-search and BLASTAp, respectively. The search results of the two methods were combined, then the combined 125 genes were de-duplicated using DNAMAN software to obtain 53 sweet orange *bZIP* genes. The conserved domains were then verified by the online website HMMER [26] (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan, accessed on 30 July 2022), and genes that did not contain conserved structural domains were excluded, resulting in the final identification of 50 *CsbZIP* genes (see Supplementary Table S1 for details).

Characterization of the protein sequences showed that the 50 *CsbZIP* genes encoded proteins ranging from 133 (*Cs_ont_9g001610*) to 773 (*Cs_ont_3g005140*) amino acids in length, with an average length of 351 amino acids. The relative molecular weights of the proteins ranged from 15.64 (*Cs_ont_9g001610*) to 86.03 (*Cs_ont_3g005140*) kD, the protein-lipid coefficients ranged from 47.02 to 88.79, the hydrophilic mean coefficients were all less than 0, and all 50 *CsbZIP* members exhibited hydrophilicity. The hydrophobicity of amino acids is one of the main drivers of protein folding, facilitating the folding of proteins to form secondary structures, further structural domains, tertiary structures, etc., and, most importantly, the formation of alpha helices to ensure the protein's stability. The predicted secondary structure of the proteins showed that irregular coiling and α-helixes were the vital components constituting the structure of *CsbZIP* proteins, accounting for 17.09–67.25% and 18.92–77.22%, respectively, while the extended chains and β-folding accounted for a small proportion (0.00–16.17% and 0.00–6.59%, respectively). The theoretical isoelectric points ranged from 4.78 to 10.84. Twenty-three *bZIP* genes had isoelectric points greater than 7. They were alkaline proteins, and the remaining proteins were acidic proteins. The protein instability coefficients of *Cs_ont_7g028150* and *Cs_ont_8g027900* were less than 40, indicating that they were stable proteins, and the rest of the family members had instability coefficients larger than 40, indicating that they were unstable proteins. In silico analysis showed that *Cs_ont_3g005140* was presumably be localized in the chloroplasts, while the rest of the family members were located in the nucleus (see Supplementary Table S2 for details).

3.2. Phylogenetic Analysis of the Sweet Orange bZIP Gene Family

To understand the evolutionary relationships among and classification of the *bZIP* transcription factor family in different species, the phylogenetic tree was constructed and embellished by the maximum likelihood tree building tool IQ-TREE after multiple sequence comparisons based on 291 *bZIP* amino acid sequences from apple, grape, sweet orange, and *Arabidopsis* (Figure 1). The *bZIP* family genes of the four species were divided into 16 evolutionary branches [34], and the *CsbZIP* genes were distributed on 11 of them. Subgroup 8 contained the highest number of *bZIP* genes (71) and the highest distribution of *CsbZIP* genes (12). Six of the 17 evolutionary branches included *bZIP* genes from only one species, with Subgroups 1 and 2 containing only *bZIP* genes from *Arabidopsis*, Subgroups 3 and 8 containing only *bZIP* genes from sweet orange, and Subgroups 4 and 5 containing only *bZIP* genes from grape and apple, respectively.

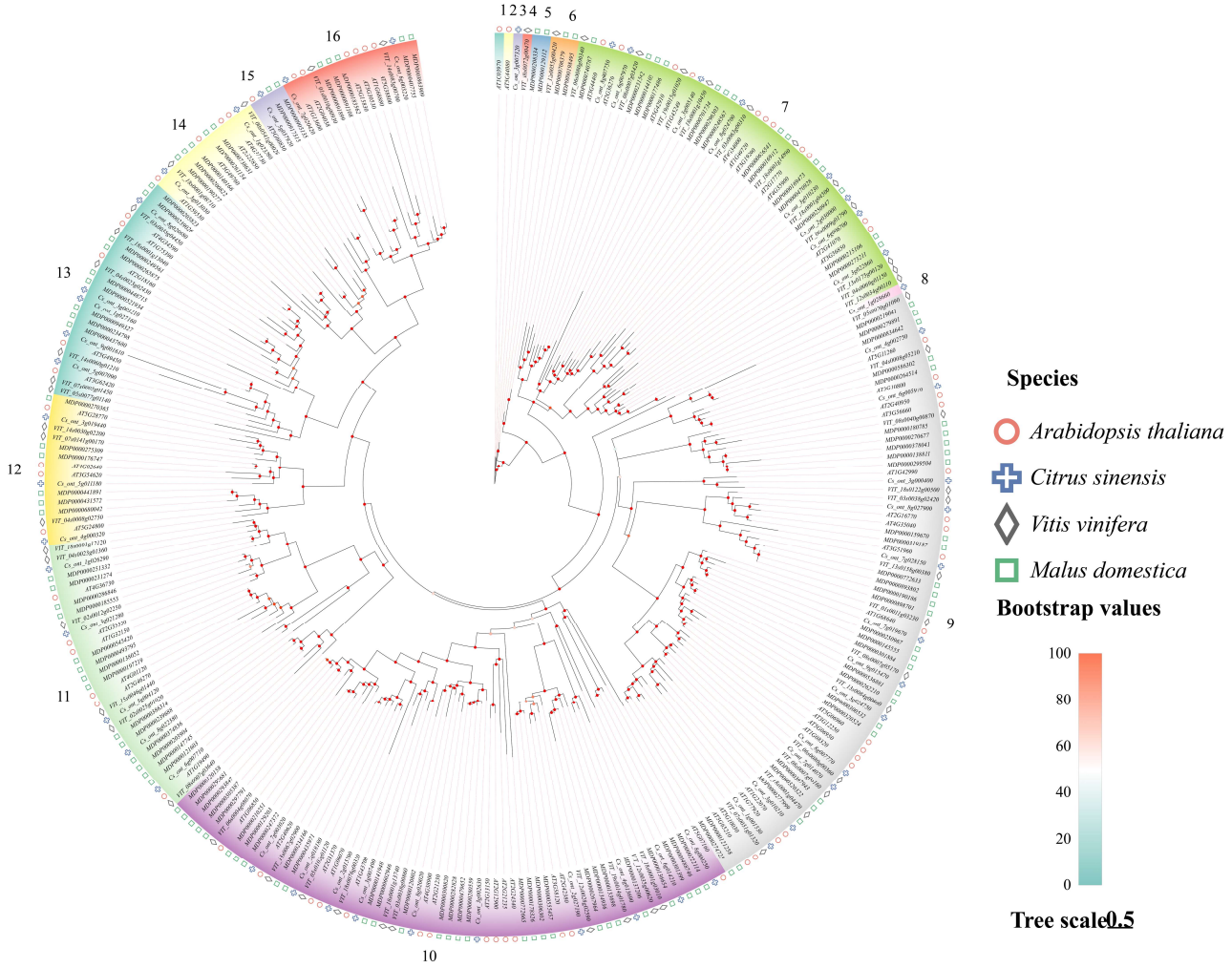
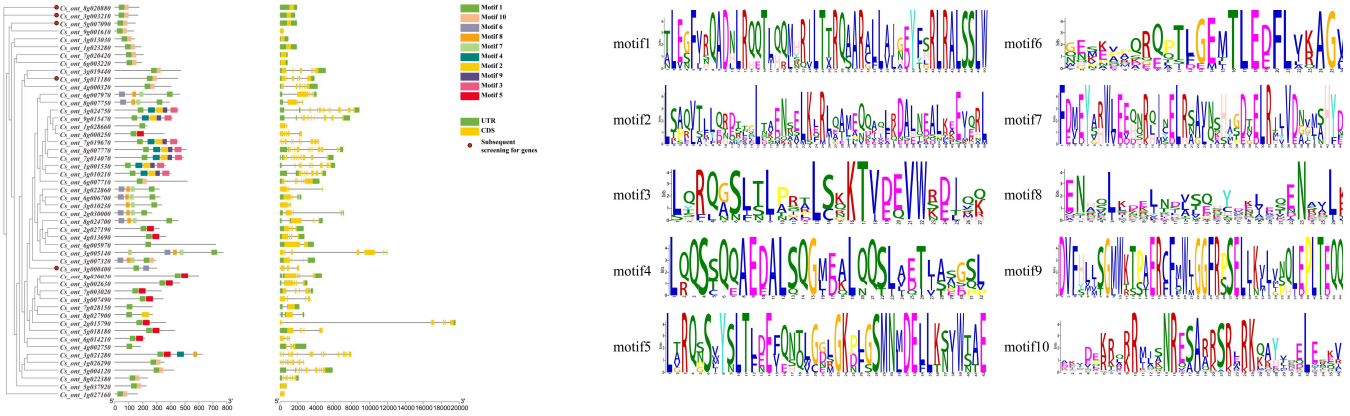


Figure 1. Evolutionary tree of the *bZIP* transcription factor family in *Malus domestica*, *V. vinifera*, *C. sinensis*, and *A. thaliana*. The evolutionary tree is based on 291 amino acid sequences encoded by *M. domestica*, *V. vinifera*, *C. sinensis*, and *A. thaliana* (see Supplementary Table S3 for details). *bZIP* genes and was constructed by IQ-TREE, a maximum likelihood tree-building software, after selecting the optimal tree-building model. The solid dots on each branch node indicate 1000 replicate bootstrap values. Red dots indicate bootstrap values of >90% in 1000 replicates, where the lighter the color of the dots, the lower the bootstrap values. The shapes on the outermost circle of the evolutionary tree represents the four different species, and the numbers 1 to 16 represent the 16 subgroups in this phylogenetic tree.

3.3. Gene Structure Analysis of the Sweet Orange bZIP Gene Family

In this study, 10 conserved motifs were identified among 50 sweet orange *bZIP* protein sequences through the online website MEME [32] and visualized using TBtools [33] software to map the motif distribution and gene structure of the *CsbZIP* gene family (Figure 2). Ten motifs were predicted by the online tool HMMER [26]. The results showed that Motif 1 had a *bZIP* conserved domain, and all sweet orange *bZIP* proteins contained motif 1. Thus, this indicated that Motif 1 is highly conserved. The conserved motif analysis showed that the number of motifs included in the sweet orange *bZIP* proteins ranged from 1 to 5, with nine *bZIP* genes containing five motifs and four *bZIP* genes containing only one motif. The type, number, and location of the conserved motifs of genes clustered in the same subfamily were alike, and *CsbZIP* and its close relatives tended to have similar gene structures. For example, the eight *CsbZIPs* distributed in Group 7 had the same motif types, four arranged in the same order (Motif 6, Motif 8, Motif 7, and Motif 1), and the

other groups also conformed to this pattern. Furthermore, Motif 3 and Motif 9 were only distributed in Group 9, so the *CsbZIP* members containing these two motifs must belong to Group 9.



(**a**) (**b**)

Figure 2. Gene structure, motif distribution, and conserved domains of the *bZIP* gene family of sweet orange. (**a**) Gene structure and motifs of the *bZIP* gene family. The phylogenetic tree of the protein sequences encoded by the 50 sweet orange *bZIP* genes, and the conserved motif composition of the proteins encoded by the *bZIP* gene family are shown on the left in Figure 2a. Different colors represent different conserved motifs, with a total of 10 motifs (Motif 1–Motif 10); the red dots mark the five genes in the follow-up screen. The gene structure of the sweet orange *bZIP* gene family is shown on the right, with yellow squares representing the coding region (CDS), the green squares representing the untranslated region (UTR), and the black lines between the squares representing the introns. The length of each gene can be estimated from the scale at the bottom. (**b**) Logos of the 10 conserved motifs of the *bZIP* gene family (see Supplementary Table S4 for details).

Exons and introns can regulate gene expression [40]. In this study, the gene structures of sweet orange *bZIPs* were analyzed (Figure 2a). The results showed that the number of introns in the sweet orange *bZIP* gene family ranged from 0 to 15, with *Cs_ont_3g021280* having the highest number of introns, namely 15, of which 10 sequences did not contain introns. In addition, *Cs_ont_3g021280* also had the highest number of exons, namely 16 exons, with 10 containing only one exon.

3.4. Analysis of the Promoter Cis-Acting Elements of the Sweet Orange bZIP Gene Family

A cis-acting element is essential to the nucleotide sequence upstream of a gene [41]. The acting element binds to the functional gene and functions as a transcription factor. To further investigate the potential mechanisms of the sweet orange *bZIP* gene family in stress responses, cis-acting element prediction was performed on their promoters. In this study, a 2000 bp nucleotide sequence upstream of the coding region of the sweet orange *bZIP* gene was extracted, and the functional elements that played a role were classified (Figure 3) as light response elements, phytohormone response elements, elements related to the regulation of plant growth and development, and stress response elements. The largest number of phytohormone response elements (326) included abscisic acid (ABA) response elements (ABRE), auxin response elements (ARE, GC-motif), gibberellin (GA) response elements (GARE-motif, P-box, TATC-box), methyl jasmonate (MeJA) response elements (CGTCA-motif, TGACG-motif, TCA-element), and flavonoid biosynthesis gene regulatory elements (MBSI), followed by light-responsive elements (LTR) (196). Four *bZIP* genes did not contain light-responsive elements (*Cs_ont_1g001530*, *Cs_ont_7g019670*, *Cs_ont_7g020420*, and *Cs_ont_8g000250*). Related elements (86) that regulate plant growth and development included cell cycle regulatory elements (MSA-like), circadian regulatory elements (circadian), en-

dosperm expression elements (AACA_motif, GCN4_motif), meristem expression-related action elements (CAT-box), elements involved in fenestra elements (HD-Zip 1), regulatory elements related to the downregulation of phytochrome expression (PDRE Unnamed_1), seed-specific regulatory elements (RY-element), and regulatory elements of maize protein metabolism (O_2-site). The stress-responsive elements (227) mainly included elements related to anaerobic induction (GC-motif, ARE), elements that respond to drought stress (MBS) [42], elements that participate in the defense and stress response (TC-rich repeats), low-temperature response elements (LTR), and wound response elements (WUN-motif).

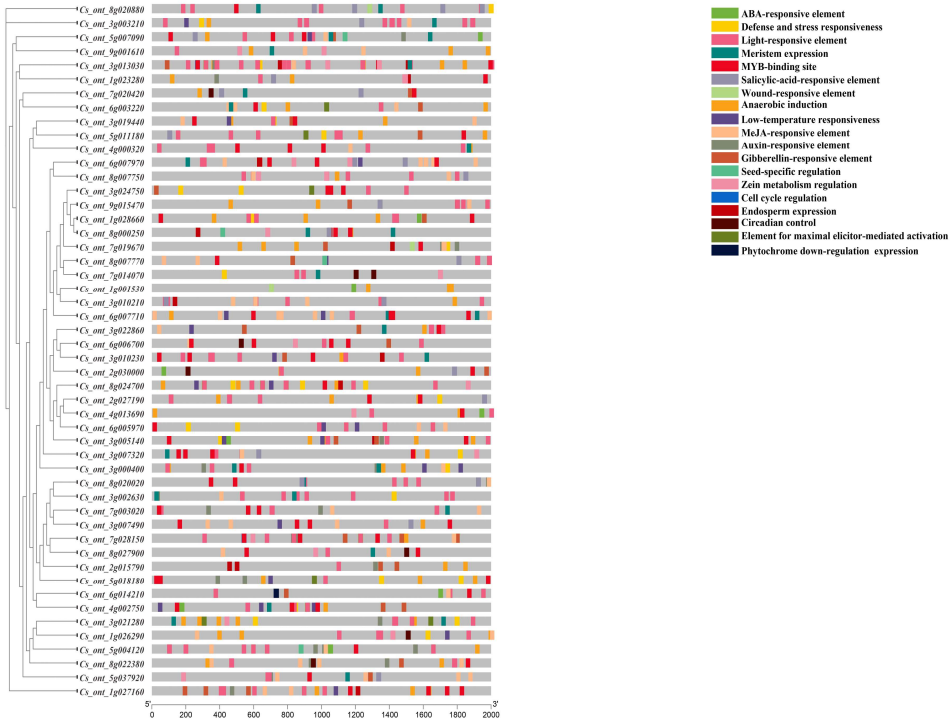


Figure 3. Predicted cis-acting elements in the promoter of the *bZIP* gene family of sweet orange. The left-hand diagram is a phylogenetic tree of the protein sequences encoded by the 50 sweet orange *bZIP* genes; the right-hand column is an analysis of the promoter cis-acting elements of the sweet orange *bZIP* genes. The different colors represent the different promoter cis-acting elements, and there are 19 promoter cis-acting elements (see Supplementary Table S5 for details).

Of the 803 promoter cis-acting elements, most phytohormone response elements were very widely distributed, with 49 sweet orange *bZIP* genes containing phytohormone response-related elements, representing 98% of the total. There were 35 (70%) sweet orange *bZIP* genes containing ABRE elements, 17 (34%) auxin response elements, 23 (46%) GA response elements, 31 (62%) MeJA response elements, and 18 (36%) SA response elements. In addition, the MBS elements associated with drought induction were distributed on 39 *CsbZIP* genes (78%), and 17 *CsbZIP* genes contained LTR elements associated with low-temperature adaptation (34%). It has been shown that ABA, SA, and MeJA play a prime role in mediating defense responses against pathogenic and abiotic stresses, and the predicted results for promoter-acting elements suggest that the vast majority of members of the *CsbZIP* gene family are involved in phytohormone signaling, as well as biotic and abiotic stress-related biological processes [43].

3.5. Analysis of Gene Covariation and Chromosomal Localization in the Sweet Orange bZIP Gene Family

The results of chromosome localization in Figure 4 show that all the identified sweet orange *bZIP* genes were on nine chromosomes, with 50 *bZIP* genes unevenly distributed on them. Chromosome 9 contained the fewest *bZIP* genes, with only

two *bZIP* genes, and chromosome 3 contained the most *bZIP* genes (13), suggesting that sweet orange's chromosome 3 may be a vital *bZIP* gene resource. Ten duplicate gene pairs were identified in the 50 *CsbZIP* genes (*Cs_ont_1g023280/Cs_ont_3g013030*, *Cs_ont_1g027160/Cs_ont_8g020880*, *Cs_ont_2g027190/Cs_ont_4g013690*, *Cs_ont_3g005140/ Cs_ont_8g024700*, *Cs_ont_3g003210/ Cs_ont_8g020880*, *Cs_ont_5g037920/Cs_ont_7g020420*, *Cs_ont_5g007090/ Cs_ont_9g001610*, *Cs_ont_6g003220/Cs_ont_7g020420*, *Cs_ont_6g007970/ Cs_ont_8g007750*, and *Cs_ont_6g014210/ Cs_ont_8g000250*), where *Cs_ont_8g020880* had gene duplications with two genes at the same time (*Cs_ont_1g027160* and *Cs_ont_3g003210*). In the gene covariance analysis, which measures how much variables change together, *Cs_ont_8g020880* was a duplicated gene pair with *Cs_ont_1g027160* and *Cs_ont_3g003210*, but *Cs_ont_1g027160* was not expressed in the control and treated groups and was probably silent, whereas *Cs_ont_3g003210* and *Cs_ont_8g020880* had gene duplications, both showing high expression, suggesting that the functions of the duplicated and re-differentiated *CsbZIP* homologs (*Cs_ont_1g027160* and *Cs_ont_3g003210*) would be further differentiated, with one responding to *Penicillium* inoculation and the other not doing so. The genes in the duplication events were mostly distributed in gene-dense regions of the chromosome, accounting for about 95%. Combining these results with those of the analysis of the evolutionary relationship analysis indicates that the pair of genes in the gene duplication event was from the same subfamily. In line with the clustering analysis results, there were cases where multiple genes are on the same chromosome in Subclades 7, 9, and 10. For example, in Subclade 7, three genes (*Cs_ont_3g005140*, *Cs_ont_3g010230*, and *Cs_ont_3g022860*) were located in the same subclade and were localized on chromosome 3. This suggests that the three genes are highly homologous and may have similar functions.

3.6. Analysis of the Tissue Expression of Sweet Orange bZIP Gene Family

Transcriptomic data were processed by Trim-galore and ***Trimmomatic*** software, and the data were checked using FastaQC to ensure that the data obtained were reliable. Transcriptome sequencing yielded over 460 million high-quality reads, with data from over 7 million high-quality genes obtained per replicate. Over 92% of the high-quality sequences generated by sequencing could be mapped to the reference genome (*C. sinensis* genome v3.0).

The transcriptomic data from *Penicillium*-infected sweet orange fruits and the control group were used to map and analyze the expression profile of the sweet orange *bZIP* gene family in the fruit (Figure 5), expressed as $\log_2$(FPKM + 1). As can be seen from the figure, 11 of the 50 *CsbZIP* genes were not expressed, 12 were upregulated, and 27 were downregulated. Among the upregulated genes, *Cs_ont_3g000400*, *Cs_ont_3g003210, Cs_ont_5g007090, Cs_ont_5g011180,* and *Cs_ont_8g020880* were highly expressed. *Cs_ont_8g020880* was a duplicate gene pair with *Cs_ont_1g027160* and *Cs_ont_3g003210* in the gene covariance analysis, but *Cs_ont_1g027160* was not expressed in either the control or treated groups, and was probably silent, while *Cs_ont_3g003210* and *Cs_ont_8g020880* had gene duplication and both showed high expression. Three of the five highly expressed genes contained the same motifs, and their gene structure was distributed in Subgroup 13. In line with the results of the promoter cis-acting elements, these genes were found to contain response elements such as MYB, LTR, ARE, SA, and MeJA phytohormone response elements. Salicylic acid (SA) and methyl jasmonate (MeJA) play a significant role in inducing increased plant stress resistance when plants were subjected to adversity and stress, which is of great significance for plants to resist the damage caused by adverse factors. On the basis of this, we hypothesized that the sweet orange *bZIP* gene, which exhibited high expression after the infestation of sweet orange fruit, may be related to the biosynthesis and metabolic pathways of salicylic acid and methyl jasmonate. The five highly expressed *CsbZIP* genes can be used as candidates for resistance to biotic stresses (*Penicillium* infestation).

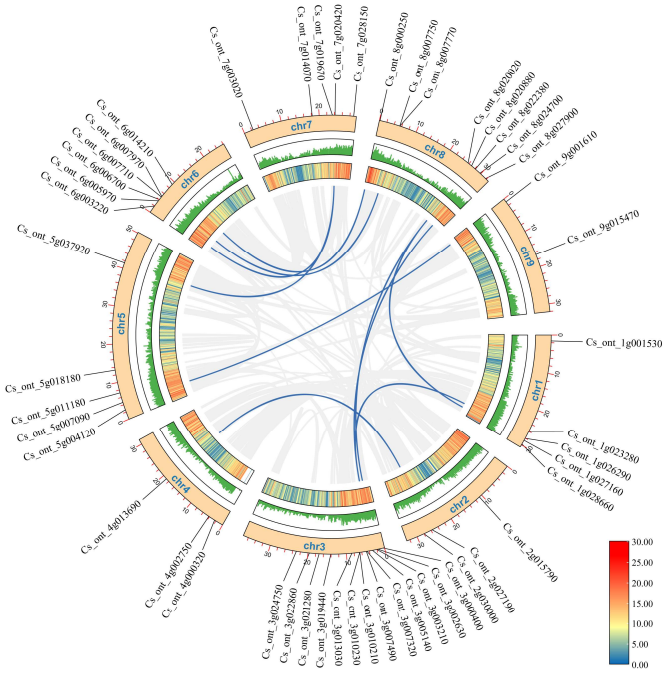


Figure 4. Chromosomal distribution of the genes of the sweet orange *bZIP* family. The grey line represents whole-genome duplication, and the two genes connected by the blue line are duplicated gene pairs. From inside to outside, the first layer of circles represents the gene density heat map, the second layer represents the gene density histogram, and the outermost layer represents the localization distribution of the sweet orange *bZIP* genes on the chromosomes.

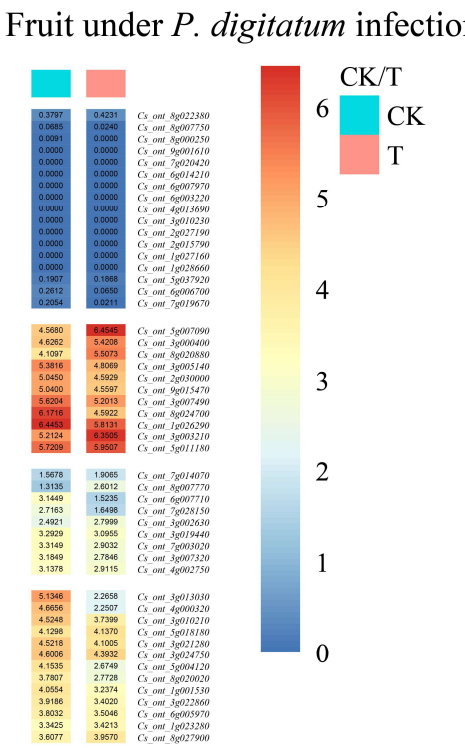


Figure 5. Gene expression profile of the *bZIP* gene family in sweet orange fruit infected by *Penicillium digitatum*. T is the treatment group, and CK is the control group. The 50 sweet orange *bZIP* genes were classified into four categories with $\log_2$(FPKM + 1) expression values, with blue representing low gene expression and red indicating high gene expression.

4. Discussion

Sweet orange has the highest proportion of citrus fruit in the world, accounting for more than half of the annual production of citrus. It is planted widely in China and has a high economic value. *bZIP* genes have been identified and studied in many plant species, and no relevant reports of *bZIP* genes have been reported in sweet orange. With the rapid development of high-throughput sequencing technology, whole-genome sequencing has been completed in many species, laying a foundation for the subsequent functional screening of genes. A growing number of studies have shown that *bZIP* genes regulate various biological and physiological processes in plant growth and development, as well as responses to biotic and abiotic stresses. The *bZIP* gene family is one of the most numerous transcription factor families in eukaryotes, and its members play vital roles in stress responses, secondary metabolism, plant growth, and seed development. Studies of these genes have been reported in several species, such as *A. thaliana* [10], *A. mume* [11], *H. brasiliensis* [12], *Pyrus communis* [44], *Gossypium hirsutum* [45], and other plants, and studies have investigated the relationship between their growth-related and biotic and abiotic stresses.

The *bZIP* gene family plays a vital role in biotic and abiotic stresses in plants. The *BpbZIP1* protein sequence was compared with several protein sequences in *Arabidopsis* and tamarisk, and the results showed that the *BpbZIP1* protein is evolutionarily related to *Arabidopsis AtbZIP12*, *AtbZIP14*, *AtbZIP27*, and *AtbZIP67*, among which, the *AtbZIP12* protein, which is the closest relative to the *BpbZIP1* protein, plays a vital role in abiotic stress [16]. This indicated that the *BpbZIP1* protein also has a role in stress tolerance. *Cs_ont_6g006700* is closely related to *AtbZIP12* and, in this study, was tentatively hypothesized to have a vital role in sweet oranges in response to salt stress and to exhibit resistance to stress. Finkelstein et al. (2000) showed that the abscisic acid response gene *ABI5* in *Arabidopsis* encodes an alkaline leucine zip transcription factor that reduces salt sensitivity during plant germination, and *Cs_ont_6g007970* is closely related to *AT2G36270* [46]. We compared the similarity between these two pairs of sweet orange *bZIP* genes and *Arabidopsis bZIP* genes, with percentages of similarity of 48% and 49%. Sung Chul Lee et al. showed that the *CAbZIP1* transcription factor functions as a possible regulator in enhanced disease resistance and environmental stress tolerance [47]. The results of Li et al. confirmed the vital role of *CsBZIP40* in improving resistance to citrus rot through the SA signaling pathway [20].

As a prime postharvest disease of citrus fruits, *Citrus Penicillium* causes considerable economic losses in storage and transport [48]. In previous studies, two phytohormones, MeJA and SA, have been shown to have vital regulatory roles in the mechanism of inducing resistance to cyanobacteria in fruit [43]. Wang et al. (2022) showed that MeJA might slow down the induction of resistance to cyanobacteria in navel orange fruit by stimulating the activity of defense enzymes and disease process-related proteins, reducing malondialdehyde content and delaying membrane lipid peroxidation [49]. Bian et al. (2019) showed that SA affected the phenol-propane metabolic pathway by inducing apples to increase the activity of fruit defense enzymes, regulating the antioxidant system in the fruit, which in turn abduction an increase in free radical scavenging, reduced malondialdehyde content in apples and reduced the diameter of bruise spots, thus preventing the occurrence of a post-harvest bruise in apples [50]. The signaling pathways mediated by SA act mainly during biotrophic and hemibiotrophic pathogen attacks, and determine the establishment of the so-called systemic acquired resistance. The signaling cascades mediated by JA and ET usually respond to necrotrophic pathogens, insects, herbivores, and injury. Extensive crosstalk between these different signal transduction pathways allows the plant to fine-tune its defenses against different types of pathogens and insect attackers. Acting as regulators of SA signaling, one class of *bZIP* proteins linked to biotic stress responses comprises the TGA cis-element-binding proteins. In a prediction analysis of the promoter cis-acting elements, the highest proportion of elements was found for phytohormone responses, including MeJA response elements (accounting for 62%) and SA response elements (accounting for 36%). All MeJA elements contained half the number of TGA-type ones and proteins that played a

role in plant defense, xenobiotic stress responses, and development [51]. Combining these results with those of the analysis of tissue expression, we inferred that *CsbZIP* activates relevant defense mechanisms after sweet orange is infected by *Penicillium*, and achieves self-defense through strong expression of the genes in response to *Penicillium* stress.

In this study, 50 Cs*bZIP* genes were identified in sweet oranges, similar to the number of *bZIP* members in *Monocotyledonous roses* [52], *Hevea brasiliensis* [31], and *Prunus persica* [53,54]. Analysis of the physicochemical properties showed that the *CsbZIP* genes encoded proteins ranging from 133 to 773 amino acids in length and 15.64 to 86.03 kD in relative molecular weight, all of which were hydrophilic. In silico analysis showed that *Cs_ont_3g005140* was presumably be localized in the chloroplasts, while the rest of the family members may be located in the nucleus, indicating that the members of *CsbZIP* mainly played a role in the previous location. Meanwhile, the transcription factors of *Cs_ont_3g005140* may play a specific role in the chloroplasts, which is similar to a report on *Vernicia fordi* [55]. The phylogenetic relationships indicated that the *CsbZIP* genes are divided into 16 subclades. Most members in the same subclade had uniform introns and conserved motifs. Three of the five highly expressed genes obtained in our screen were from Subclade 13 and were closely related evolutionarily. They shared the same conserved motif species and alignments, and are therefore inferred to have similar gene functions. A predictive analysis of the promoter cis-acting elements showed that the functional acting elements can be classified into four categories: light-responsive elements, phytohormone-responsive elements, elements related to the regulation of plant growth and developmental, and stress-responsive elements. The phytohormone response elements were the most numerous and had the highest percentage. Previous studies have shown that phytohormones such as ABA, SA, and MeJA play a prime role in mediating defense responses to pathogenic and abiotic stresses [43,56]. The high proportion of phytohormone response elements indicated that most of the *CsbZIP* family members are involved in the synthesis or signaling of phytohormones, and are closely related to the biotic (abiotic) stress response of plants. The results of chromosome localization showed that all 50 *CsbZIP* genes identified were anchored on nine chromosomes, with a heterogeneous distribution. The number of genes distributed on each chromosome was not related to the length of the chromosomes, and chromosome 3 had the highest number of genes distributed on it, and it may contain vital genetic resources of sweet orange *bZIP*. The covariance analysis showed that there were 10 duplicate gene pairs in the sweet orange *bZIP* gene family, and almost all of these duplicates were located in the gene high density region of the chromosome, accounting for up to 95% of the total. *Cs_ont_8g020880* and two genes had gene duplication, so two copies were present (*Cs_ont_1g027160* and *Cs_ont_3g003210*). The gene expression profile of the *bZIP* gene family of *Penicillium*-infected sweet orange fruits showed that 39 genes were expressed and 11 genes were not, of which five genes were highly expressed (*Cs_ont_3g000400, Cs_ont_3g003210, Cs_ont_5g007090, Cs_ont_5g011180*, and *Cs_ ont_8g020880*). Combined with the results of covariance analysis, this showed that the set of duplicated gene pairs *Cs_ont_8g020880* and *Cs_ont_3g003210* were significantly expressed in the expression profile, while the other copy of *Cs_ont_8g020880* was not in the profile, and there may be gene silencing.

5. Conclusions

For the first time, the *bZIP* gene family in the whole sweet orange genome was identified and analyzed using bioinformatics methods, and a total of 50 *CsbZIP* genes were obtained.

Cs_ont_6g006700 is closely related to the *Arabidopsis AT2G41070*(*AtbZIP12*) gene, which plays a vital role in abiotic stress; *Cs_ont_6g007970* is closely related to *AT2G36270*, which reduces plants' sensitivity to salt. We compared the similarity between these two pairs of sweet orange *bZIP* genes and *Arabidopsis bZIP* genes, with percentages of similarity of 48% and 49%, respectively.

There were 10 duplicate gene pairs in the *CsbZIP* gene family, of which *Cs_ont_8g020880* had two copies (*Cs_ont_1g027160* and *Cs_ont_8g020880*). The functions of the replicated and re-differentiated *CsbZIP* homologs diverged further, with one responding to *Penicillium* inoculation and the other not doing so.

Five *CsbZIP* genes (*Cs_ont_3g000400*, *Cs_ont_3g003210*, *Cs_ont_5g007090*, *Cs_ont_5g011180*, and *Cs_ont_8g020880*), which are highly expressed during *Penicillium* infestation of sweet orange, were initially screened as candidate genes for resistance to *Penicillium* infestation in sweet orange.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9030393/s1**,** Table S1. The information on the *bZIP* gene family in sweet orange. Table S2. Physicochemical properties, subcellular localization, and secondary structure of the sweet orange *bZIP* gene family. Table S3. The information on the *bZIP* gene family in *Citrus* sinensis, Arabidopsis thaliana, Malus *domestica and Vitis vinifera*. Table S4. Detailed information on identified ten motifs in *bZIP* proteins. Table S5. Information on cis-elements detected in the promoter regions of *bZIP* genes.

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Data Availability Statement: The C. sinensis genome (v3.0) and annotation files of sweet orange are openly available in the CPBD: Citrus Pan-genome to Breeding Database (https://citrus.hzau.edu.cn/download.php). RNA-Seq data were presented at the short read archive (SRA) database of the National Center for Biotechnology Information (NCBI, accession number: SRR19976158, SRR19976159, SRR19976157, SRR19976160, SRR19976161, SRR19976156). The other data presented in this study are available in Supplementary Materials.

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