RESEARCH ARTICLE

Genome-wide analysis and expression profile of *bZIP* gene family in *Pinellia ternate*

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Pinellia ternate **is an important medicinal plant. During its growth process, it is extremely sensitive to temperature and light and is prone to seedling collapse phenomenon, in which can reduce its yield. *bZIP* gene family plays a key role in governing the development and morphogenesis of the plant as well as its responses to both biotic and abiotic stresses. This research investigated the characteristics of the members of *bZIP* gene family and evaluated their potential functions in stress-resistant breeding through genome-wide analysis on *P. ternate bZIP* gene family using multiple databases and online resources to explore phylogenetic relationships, gene structure, chromosomal localization, conserved motifs, cis-regulatory components, transcriptional expression patterns, and possible interactions among proteins. The results showed that *bZIP* gene family in *P. ternate* consisted of 35 members named *PtbZIP*. *PtbZIP* genes encoded proteins with different lengths from 111 to 711 amino acids. All these proteins presented hydrophilic properties and were localized within the nucleus. The 13 chromosomes of *P. ternate* were unequally distributed in *PtbZIP* genes. Based on the results obtained from phylogenetic studies, *PtbZIP* genes could be divided into seven sub-families with genes within identical groups presenting comparable structural properties. The conserved motifs were identified in all *PtbZIP* genes. Collinearity analysis revealed 11 duplication events, 5 of which were identified as tandem duplication events. Examination of *PtbZIP* gene expression patterns in *P. ternata* under high-temperature stress showed that 8 genes presented gradual upregulation, while those of 11 genes were declined with the extension of heat stress duration. It was also found that *PtbZIP* gene could respond to high-temperature stress and might be involved in collapse regulation process. These insights enhanced *PtbZIP* comprehension and established a significant foundation for subsequent functional analysis on *PtbZIP* genes in *P. ternata*.**

Keywords: *bZIP*; *Pinellia ternate*; gene family; bioinformatics; expression pattern.

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Introduction

Pinellia ternata (Thunb.) Breit. is a tuberous plant and a member of genus *Pinellia* within *Araceae* family [1]. It flourishes in environments with warm climates, nutrient-rich soil, and abundant water, making the regions of Henan, Shandong, and Hebei provinces in China particularly suitable for its cultivation [2, 3]. Research has identified a variety of chemical constituents in *Pinellia tubers* including alkaloids, volatile oils [4], flavonoids [5], fatty acids [6], and organic acids [7]. Among them, alkaloids are the main bioactive compounds with at least 25 identified

components including L-ephedrine, choline, adenosine, guanosine, and thymidine [8], which present pharmacological properties such as antiemetic [9], analgesic, antiarrhythmic, and antitumor effects [10]. There is a significant demand for *P. ternata* with an estimated annual consumption of about 6,000 tons [11]. Temperature plays a key role in determining both yield and growth since extreme temperatures, no matter excessively high or low, have adverse effects on plant development. Particularly, elevated summer temperatures can contribute to seedling collapse and yield reduction. Therefore, at high-temperatures, prevention of *P. ternate* collapse is essential to increase yield. However, relatively few reports are available on the molecular mechanisms that alleviate *P. ternate* seedling death under high-temperature conditions.

High temperatures affect crop growth and development and decrease crop yields [12]. Transcription factors (TF) are essential for several functions such as stress resistance responses, metabolic reactions, and plant growth and development [13]. Research has found that *bZIP* gene family plays a role in plants under high-temperature stress [14]. In maize, the *bZIP60* gene can activate the expression of the key heat shock protein (HSP) gene *HSFTF13*, which in turn upregulates the expression of heat-stress response (HSR) genes and promotes HSR. In contrast, the *bZIP60* mutant suppresses the normal upregulation of HSP genes under high temperature, resulting in the attenuation of HSR under heat stress conditions [15]. Under high-temperature treatment, expression levels of *Dimocarpus longan bZIP* [16], maize *bZIP26* [17], *Panicum virgatum bZIP* [18], and *Arabidopsis thaliana bZIP18* and *bZIP52* [19] were all increased. Under normal conditions, *AtbZIP18* and *AtbZIP52* were in cytoplasm, but heat stress re-localized them to nucleus, thereby jointly regulating the expression of corresponding genes. Furthermore, overexpression of *bZIP* gene significantly enhanced the high temperature tolerance of transgenic plants [19]. In the context of global warming, the application of *bZIP* members capable of resisting high temperatures to improve crop quality and increase crop yield is of great significance for modern agricultural production.

Currently, the chromosome-level genome of *P. ternata* has been successfully assembled [1]. This research performed a comprehensive whole-genome analysis of *bZIP* gene family in *P. ternate*, providing valuable references for the molecular mechanism of the collapse of *P. ternate* under high-temperature stress. This study holds significant implications for the cultivation of high-quality *Pinellia* varieties.

Materials and methods

Detection of *bZIP* genes within *P. ternata*

The genome and annotation file of *P. ternata* were accessed in the Chinese National Center of Bioinformation (https://ngdc.cncb.ac.cn/gwh/Assembly/37791/show). Protein annotation of *P. ternata* was performed using PfamScan to provide the corresponding annotation document [20]. To obtain the gene identifier labeled *bZIP*, *bZIP* gene was associated with Pfam identifier PF00170 (https://www.ebi.ac.uk/interpro/). For the validation of *bZIP* genes, bZIP protein sequences were isolated and used to perform domain identification analyses using InterPro (https://www.ebi.ac.uk/interpro/). *PtbZIP* was generated by conducting BLASTP and BLASTN tests (sequence recognition rate = 80% and query length coverage = 50%). The molecular properties of PtbZIP proteins including isoelectric point, molecular mass, and grand average of hydropathicity (GRAVY) were evaluated using ExPASy (http://web.expasy.org/protparam/). Cell-PLoc 2.0 platform (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) was applied for the prediction of subcellular distribution.

Multiple sequence alignment and evolutionary analyses

The sequence *A. thaliana* bZIP protein was obtained from TAIR database (http://www.arabidopsis.org). Neighbor-joining

(NJ) approach was applied to generate PtbZIP-AtbZIP phylogenetic tree using MEGA X software [21]. To ensure statistical robustness, the bootstrap parameter was set up with 1,000 repeats. The visualization of the phylogenetic tree was enhanced using EvolView software (https://www.evolgenius).

Chromosome localization

To ensure the chromosomal positions of the genes and obtain corresponding chromosomal physical maps, TBtools program [22] was integrated with the whole-genome annotation file and PtbZIP gene family.

Collinearity analysis

MCScanX software with default settings was applied to analyze PtbZIP gene duplication in *P. ternata*. Collinearity of *P. ternata* was investigated *via* TBtools software using one-step MCScanX command.

Conservative motif and gene structure analyses

Gene structure display server (GSDS) platform was employed to study the exon-intron architecture of PtbZIP protein sequences (http://gsds.gao-lab.org/index.php). MEME software (http://meme-suite.org/tools/meme) was applied for the identification and analysis of the conserved domains of PtbZIP proteins, maintaining default parameters while setting motif number to 10 [23]. Positional information of PtbZIP proteins was extracted and presented using TBtools software, which also facilitated the graphical analysis of structural characteristics, phylogenetic relationships, and motif patterns of PtbZIP proteins. Chiplot was applied to analyze the domain of PtbZIP protein.

Selective pressure analysis

PtbZIP gene database was developed using makeblastdb command. BLASTN was used for the comparison of *PtbZIP* nucleic acid sequences. Ka/Ks ratio was determined using the Ka/Ks_Calculator tool in TBtools software to explore the ratio of non-synonymous (Ka) to synonymous (Ks) substitutions. This ratio evaluated whether selective pressure was exerted on *PtbZIP*. A Ka/Ks value higher than 1 signified positive selection, while values below 1 indicated purifying selection.

Analysis of promoter regions of *P. ternata* species

The 2,000 bp segment coming before the transcriptional start point of family members was identified as the promoter region of *PtbZIP* genes. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database was applied to analyze the regulatory components, and TBtools was utilized to present the findings.

PtbZIP gene expression patterns analysis

P. ternata RNA-seq data under high-temperature stress at 0, 4, and 24 hours were obtained from National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1079994). Log2 (FPKM+1) was applied to convert this data and pheatmap R package was employed to generate a heatmap showing the expression patterns of *PtbZIP* gene.

Protein-protein interaction (PPI) network analysis

Protein interaction network was established by uploading the 35 discovered PtbZIP protein sequences to Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://cn.string-db.org) with minimum interaction score of 0.400.

Results and discussion

Characterization and examination of PtbZIP family members

A total of 35 PtbZIP protein sequences was identified from *P. ternata* genome and labelled as PtbZIP1 to PtbZIP35 (Table 1). Physicochemical analyses revealed that the length of PtbZIP proteins ranged from 111 aa to 711 aa with molecular weights ranging between 12,785.55 Da and 76,096.12 Da and isoelectric point values ranging from 5.13 to 10.18. Except for PtbZIP33, the rest of PtbZIP proteins were all unstable. The

Table 1. Features of the bZIP gene family in *P. ternate*.

Gene	Length	Molecular weight	Isoelectric point	Instability index	GRAVY	Subcellular localization
PtbZIP1	300	32,366.08	5.13	59.83	-0.607	Nucleus
PtbZIP2	512	55,688.22	6.59	71.44	-0.774	Nucleus
PtbZIP3	205	22,938.08	10.18	76.11	-0.624	Nucleus
PtbZIP4	451	48,211.37	5.74	64.65	-0.678	Nucleus
PtbZIP5	348	37,470.48	8.39	51.40	-0.901	Nucleus
PtbZIP6	422	45,377.24	6.00	62.40	-0.673	Nucleus
PtbZIP7	413	44,533.29	6.17	62.49	-0.708	Nucleus
PtbZIP8	159	17,469.85	7.89	63.84	-0.520	Nucleus
PtbZIP9	467	49,930.78	6.15	53.07	-0.794	Nucleus
PtbZIP10	479	50,965.98	6.15	52.16	-0.749	Nucleus
PtbZIP11	307	34,450.02	8.8	53.37	-0.749	Nucleus
PtbZIP12	382	41,345.00	5.99	65.78	-0.729	Nucleus
PtbZIP13	407	42,834.79	9.26	50.52	-0.360	Nucleus
PtbZIP14	111	12,785.55	9.50	55.79	-0.521	Nucleus
PtbZIP15	472	50,708.50	6.25	56.5	-0.715	Nucleus
PtbZIP16	504	54,055.31	6.30	55.91	-0.679	Nucleus
PtbZIP17	444	48,082.75	6.07	60.19	-0.704	Nucleus
PtbZIP18	350	38,167.55	5.59	50.00	-0.514	Nucleus
PtbZIP19	424	44,423.85	6.52	60.12	-0.764	Nucleus
PtbZIP20	355	38,827.31	9.15	54.42	-0.844	Nucleus
PtbZIP21	416	43,794.66	6.23	62.49	-0.891	Nucleus
PtbZIP22	711	76,080.12	6.36	49.06	-0.497	Nucleus
PtbZIP23	711	76,096.12	6.36	49.06	-0.501	Nucleus
PtbZIP24	172	19,279.68	8.36	40.98	-0.828	Nucleus
PtbZIP25	293	31,407.53	6.49	51.16	-0.898	Nucleus
PtbZIP26	345	37,833.51	6.95	65.69	-0.745	Nucleus
PtbZIP27	147	16,182.22	6.15	64.91	-0.569	Nucleus
PtbZIP28	257	28,308.74	5.68	47.42	-0.712	Nucleus
PtbZIP29	226	24,882.04	5.81	46.61	-0.692	Nucleus
PtbZIP30	379	40,853.36	6.53	53.71	-0.786	Nucleus
PtbZIP31	275	29,724.44	9.85	83.84	-0.633	Nucleus
PtbZIP32	146	16,055.12	7.93	55.61	-0.486	Nucleus
PtbZIP33	485	51,607.02	8.07	38.93	-0.386	Nucleus
PtbZIP34	182	19,728.28	9.74	80.33	-0.706	Nucleus
PtbZIP35	165	18,103.93	9.68	54.06	-1.107	Nucleus

GRAVY of all PtbZIP proteins was negative, and they were considered hydrophilic. In addition, subcellular localization analyses revealed that the predominant fraction of PtbZIP proteins was confined to the nucleus. These proteins were different in terms of molecular weight, amino acid composition, and isoelectric point.

Phylogenetic analysis of PtbZIP genes

Phylogenetic investigations found that all 35 members of *PtbZIP* family have presented significant conservation throughout evolutionary history. Based on *thaliana* classification, *PtbZIP* genes family was accurately classified into seven groups (Figure 1). The largest cluster labelled as group A comprised 11 members, whereas groups B, F, and H contained only 2 *PtbZIP* members each. *PtbZIP* genes were divided into 7 subfamilies, presenting slight differences compared to those identified in other species of *Arabidopsis* with 72 *AtbZIP* genes and 8 subfamilies, Wheat with 227 *TabZIP* genes and 13 subfamilies, and *Ricinus communis* with 49 *RcbZIP* genes and 11 subfamilies. The results suggested that *PtbZIP* genes underwent selection during domestication.

Chromosomal localization of PtbZIP genes

PtbZIP family presented an uneven distribution across 13 chromosomes of *P. ternate* and formed

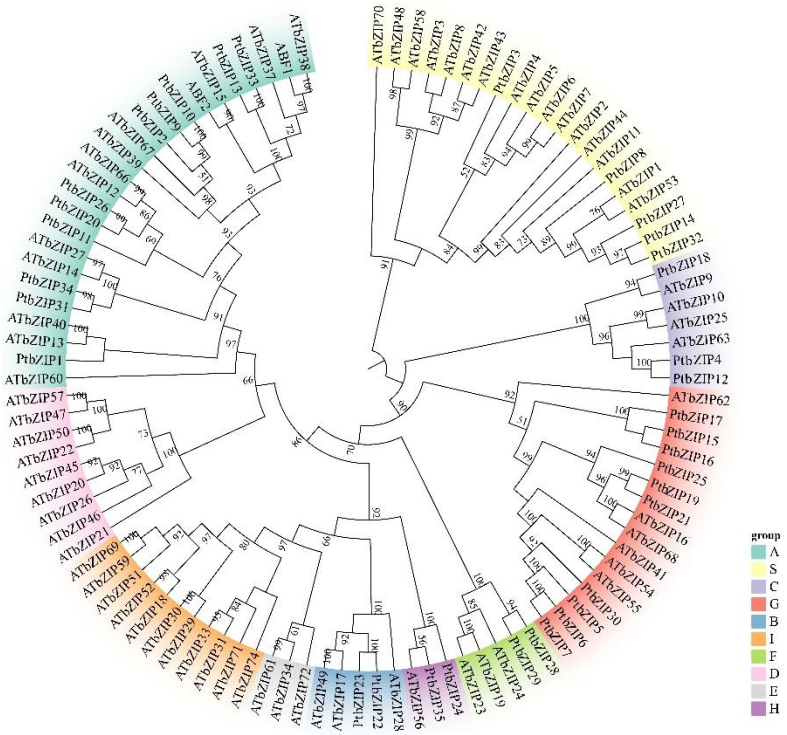

Figure 1. Phylogenetic analysis of *bZIP* genes from *P. ternata* and *A. thaliana*.

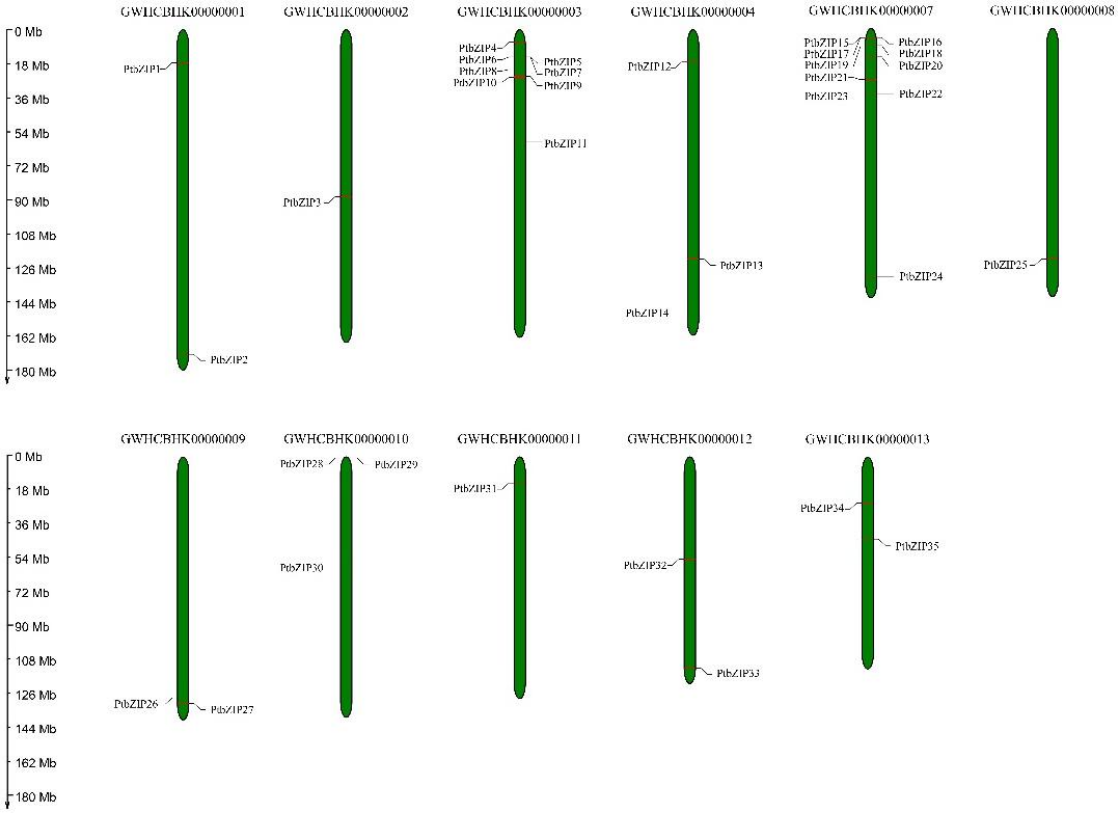

Figure 2. Chromosomal localization analysis of PtbZIP genes.

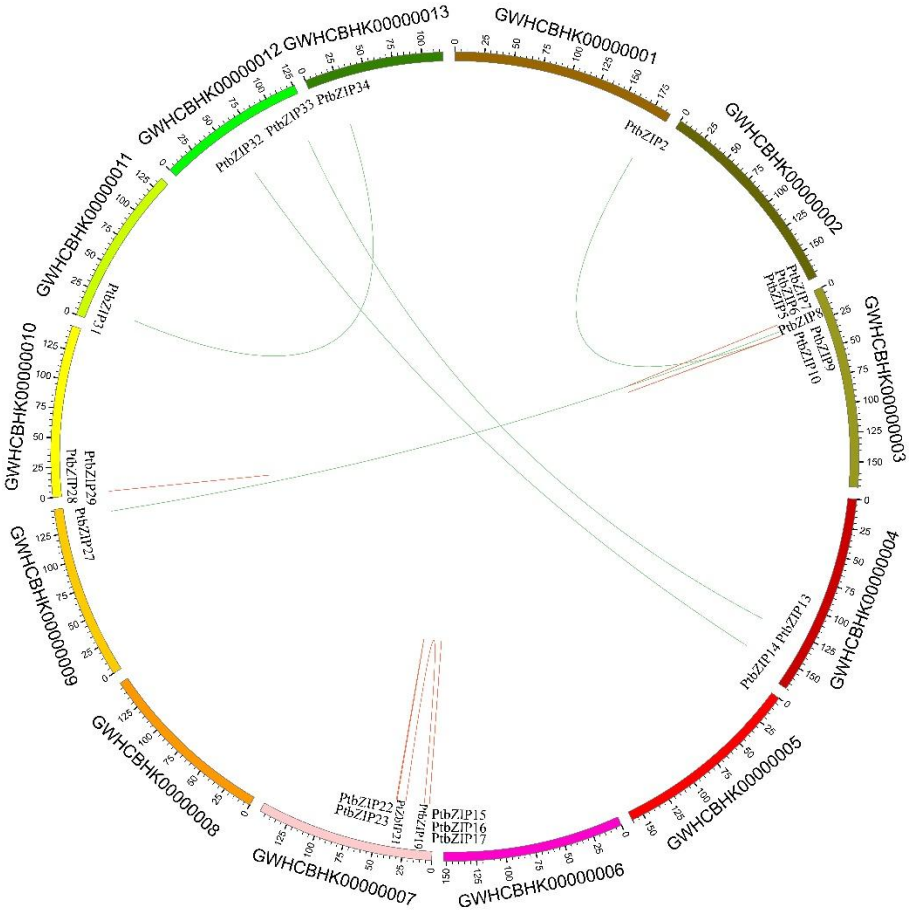

Figure 3. Collinearity of *bZIP* gene family groups in *P. ternate*.

multiple gene clusters. Among them, *PtbZIP* gene was most distributed on chromosome 7 with a total of 9 occurrences. Eight *PtbZIP* genes were distributed on chromosome 3 but no *PtbZIP* gene on chromosomes 5 and 6 (Figure 2).

Collinearity of *PtbZIP* genes

Replication events were analyzed to evaluate the potential evolutionary mechanisms of *PtbZIP* gene family. Collinear analysis within *Pinellia* genome identified 11 gene pairs exhibiting fragmental duplications, among which 5 pairs of *PtbZIP5*:*PtbZIP6*:*PtbZIP7*, *PtbZIP9*:*PtbZIP10*, *PtbZIP15*:*PtbZIP16*:*PtbZIP17*, *PtbZIP22*:*PtbZIP23*, *PtbZIP28*:*PtbZIP29* were identified as tandem duplications (Figure 3). Gene segment and tandem duplications were recognized as significant drivers of gene family expansion and performed a fundamental function in the genetic evolution of plant genomes. These genes might share similar biological functions, potentially mitigating function loss due to gene deletions or mutations.

Conserved motif analysis and exon–intron gene structure

A phylogenetic tree was generated by aligning 35 PtbZIP protein sequences using NJ technique (Figure 4A). The conserved motifs of 35 PtbZIP proteins were predicted using TBtools and MEME online program (Figure 4B). PtbZIP family members contained the conserved Motif1 with the sequence of EEKRQRRMJSNRESARRSRARKQA YTEELETQVAELKEENARLRKZJSRJ. Many PtbZIP proteins had only one Motif1 including PtbZIP3, PtbZIP4, PtbZIP8, PtbZIP12, PtbZIP14, PtbZIP18, PtbZIP24, PtbZIP27, PtbZIP31, PtbZIP32, PtbZIP34, and PtbZIP35 proteins. The results of

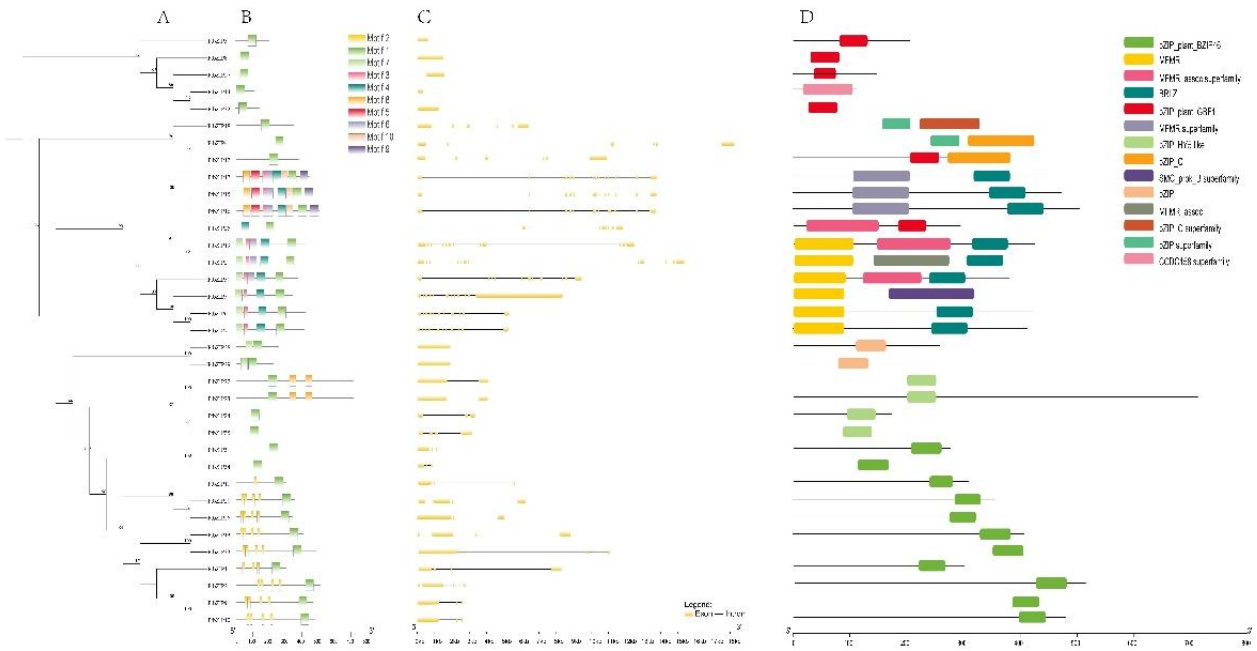

Figure 4. Conserved motifs and gene structure of *PtbZIP* genes.

comparison found that Motif1 was a typical zinc-like finger structure of CX2CX6CX3C, which was a marker of *bZIP* gene family. In addition, changes were observed in both the number and types of conserved motifs among various PtbZIP members. The numbers of genes containing Motif2 to Motif10 were 6, 9, 10, 3, 6, 8, 5, 3, and 3, respectively. The gene structures of *PtbZIP* family members were investigated to explore the evolutionary patterns of this gene family and provide significant insights into its functional diversification. The results showed that all *PtbZIP* genes contained exons ranging from 1 to 13 and intron varying from 0 to 12, presenting significant structural differences among PtbZIP family members (Figure 4C). Further comparative analysis revealed that genes positioned in close proximity within the evolutionary tree presented remarkable similarities in gene structure. Eight *PtbZIP* genes belonging to subfamily C presented high similarity with exon and intron numbers consistently maintained at 6 and 5, respectively. This variation indicted that the loss or gain of exons and introns as well as their insertion events took place throughout *PtbZIP*s evolutionary history, which might affect their biological functionality. Domain analysis results of PtbZIP demonstrated that 13 proteins had MFMR domain, 11 proteins had bZIP plant BZIP46 domain, and 8 proteins had BRLZ domain (Figure 4D). Along with the results of evolutionary tree, there was only bZIP plant BZIP46 area in group A. PtbZIP28 and PtbZIP29 only contained bZIP domain and were clustered together.

Selective pressure analysis

The influence of selection pressure during evolution on PtbZIP family components was evaluated (Table 2). Among these 6 homologous gene sequence pairs, the Ka/Ks values of 4 pairs were less than 1, indicating that most of *PtbZIP* genes had undergone strong purification selection.

Promoter analysis of *PtbZIP* genes

A promoter region of about 2,000 bp upstream of the transcription start site of *PtbZIP* family members was analyzed, revealing various cis-acting components (Figure 5). Several response elements with distinct functions were identified. Among them, 436 were light-responsive elements, indicating that this gene might be

Table 2. Divergence between paralogous *bZIP* gene pairs in *P. ternate*.

Gene1	Gene2	Ka	Ks	Ka/Ks
PtbZIP2	*PtbZIP10*	0.308020939	0.826486	0.372687
PtbZIP2	*PtbZIP9*	2.161258848	1.230359	1.756609
PtbZIP6	*PtbZIP7*	0.053833519	0.063222	0.851494
PtbZIP9	*PtbZIP10*	2.055728279	1.502970	1.367777
PtbZIP22	*PtbZIP23*	0.000617000	0.009814	0.062920
PtbZIP31	*PtbZIP34*	0.043077070	0.051777	0.831980

Figure 5. Prediction of cis-acting elements in the promoters of *PtbZIP* genes.

sensitive to light-mediated regulation. 381 elements were hormone-responsive including those associated with abscisic acid (ABA) and methyl jasmonate (MeJA). MeJA is a plant hormone with a fundamental role in plant growth and resistance to diseases and pests, while abscisic acid belongs to sesquiterpene hormone family and is an essential regulator of stress responses and growth in plants. Therefore, it could be concluded that *bZIP* genes in plants might control the expression of genes related to hormone signaling pathways through transcriptional regulation, thereby affecting the growth, development, and stress responses of the plant, and ultimately exerting a regulatory effect on *P. ternata* collapse phenomenon.

Analysis of *PtbZIP* genes expression pattern under high-temperature stress

The expression pattern of *PtbZIP* gene family in *P. ternata* under high-temperature stress demonstrated that *PtbZIP22* and *PtbZIP23* expression levels exhibited a continuous increase with prolonged exposure to high-temperature stress. However, a decrease in expression level was observed over time for 4 genes including *PtbZIP1*, *PtbZIP4*, *PtbZIP20*, and *PtbZIP26*. Furthermore, 7 genes including *PtbZIP13*, *PtbZIP32*, and *PtbZIP33* showed up-regulation at 4 hours followed by a subsequent reduction with the extension of high-temperature treatment duration. 6 genes including *PtbZIP8*, *PtbZIP12*, and *PtbZIP27* showed a decrease at 4 hours and then an increase with the extension of high-temperature treatment duration. 8 genes presented gradual upregulation, while those of 11 genes were declined with the extension of heat stress duration (Figure 6). These results suggested that *PtbZIP* genes might be involved in *P. ternata* regulatory mechanism in response to high-temperature stress and perform multiple biological functions therein. As a commonly applied bulk medicinal material in China, *P. ternata* is highly prone to excessive elongation of resulting in lodging under high-temperature conditions, resulting in reduced yield. This research provided crucial genomic resources for subsequent evaluation of the functions of *PtbZIP* genes in *P. ternata* seedling growth and development, their roles in seedling lodging regulation, and their potential applications in

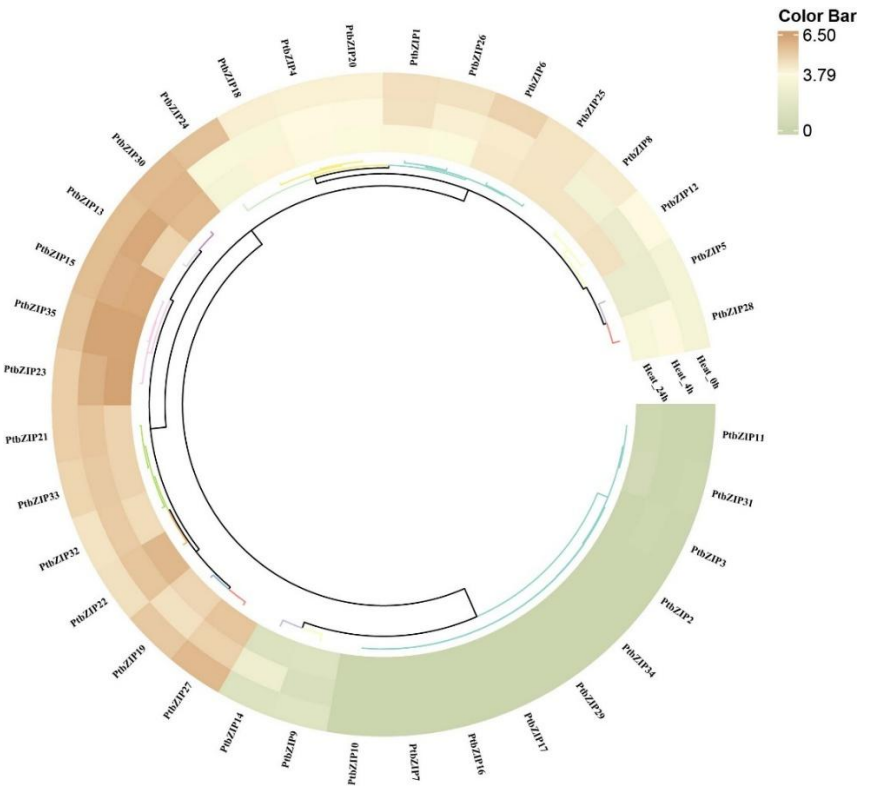

Figure 6. Analysis of *PtbZIP* gene family expression pattern.

molecular breeding.

PPI network analysis

A PPI interaction network was developed using STRING database based on *Arabidopsis* protein orthologs to evaluate interactions among *PtbZIP*s and related proteins. The sequence identities of 32 PtbZIP proteins were found to range from 30.5 to 66%, indicating *Arabidopsis* orthologs. The results showed that the PPI network contained 15 nodes and 40 edges, suggesting that these PtbZIPs interacted with one another as well as other proteins to support various biological functions (Figure 7). When exposed to light, a transcription factor HY5 protein stimulated photomorphogenesis, which regulated plant growth and development by acting on the downstream areas of the photoreceptor network and directly influencing the transcription of genes activated by light. The primary ABA-inhibited growth mediator ABI5 protein also played a role in ABA-regulated gene expression and controlled plant seed development. Research has shown that BZIP63 and AtbZIP3 might play important roles in ABA-mediated glucose transport.

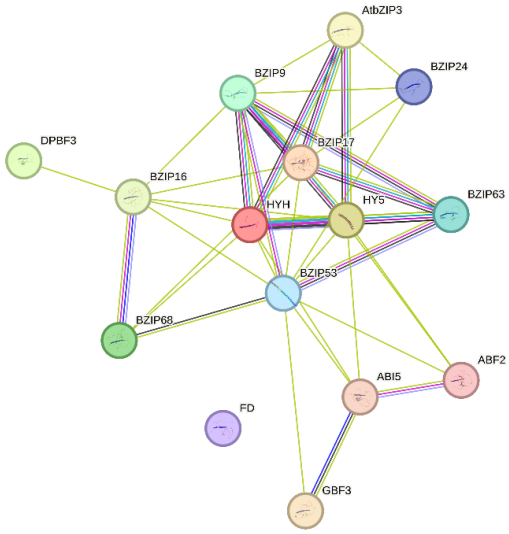

Figure 7. PPI network of significant PtbZIP proteins in *P. ternate*.

This research identified 35 *PtbZIP* genes at chromosomal level. The phylogeny, gene structure, evolution, and homeopathic components of *PtbZIP* genes were investigated. Most genes in the family were activated at high temperatures. The results were helpful to further explain *PtbZIP* gene function in the regulation of the advancement of *P. ternate* and provided reference for genetic breeding.

Acknowledgements

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Data availability

The *P. ternata* genome assembly and annotation data are available at China National Center for Bioinformation (https://ngdc.cncb.ac.cn/gwh/Assembly/37791/show). Transcriptome data of *P. ternata* treated with high temperature are available at National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/bioproject/1079994).

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