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Characteristic analysis of BpbZIP family genes and *BpbZIP26* significantly enhanced triterpenoid production in *Betula platyphylla* under S-nitrosothiol treatment

Bo Wang†, Xinglei Gao, Haixin Yang†, Huimin Liu and Guizhi Fan*

Abstract

Background: Basic leucine zipper (bZIP) transcription factors are crucial in plant development, and response to environmental stress, etc. With the development of sequencing technology and bioinformatics analysis, the bZIP family genes has been screened and identified in many plant species, but bZIP family genes has not been systematically characterized and identified their function in *Betula platyphylla*.

Methods: *B. platyphylla* reference genome was used to characterize bZIP family genes. The physicochemical properties, chromosome distribution, gene structure, and syntenic relationships were analyzed by bioinformatics methods. The effect of *BpbZIP26* on triterpenoid production was investigated using *Agrobacterium*-mediated transient transformation under N6022 treatment.

Results: 51 *bZIP* family genes were identified in *B. platyphylla*, and named *BpbZIP1–BpbZIP51* sequentially according to their positions on chromosomes. All *BpbZIP* genes were unevenly distributed on 14 chromosomes, and divided into 13 subgroups according to the classification of *Arabidopsis thaliana* bZIP proteins. 12 duplication events were detected in the *B. platyphylla* genome, and 28 orthologs existed between *B. platyphylla* and *A. thaliana,* 83 orthologs existed between *B. platyphylla* and *Glycine max*, and 73 orthologs existed between *B. platyphylla* and *Populus trichocarpa*. N6022 treatment changed gene expression levels of most *BpbZIPs* in seedlings of *B. platyphylla*. Among of them, N6022 treatment significantly enhanced gene expression levels of *BpbZIP26* in leaves, stems and roots of *B. platyphylla*. *BpbZIP26* mediated triterpenoid production, and N6022 treatment further enhanced triterpenoid production in *BpbZIP26* overexpression calli of *B. platyphylla* using *Agrobacterium*-mediated transient transformation.

Conclusion: This work highlights potential *BpbZIP* family genes responding to S-nitrosothiol and provides candidate genes for triterpenoid production in *B. platyphylla*.

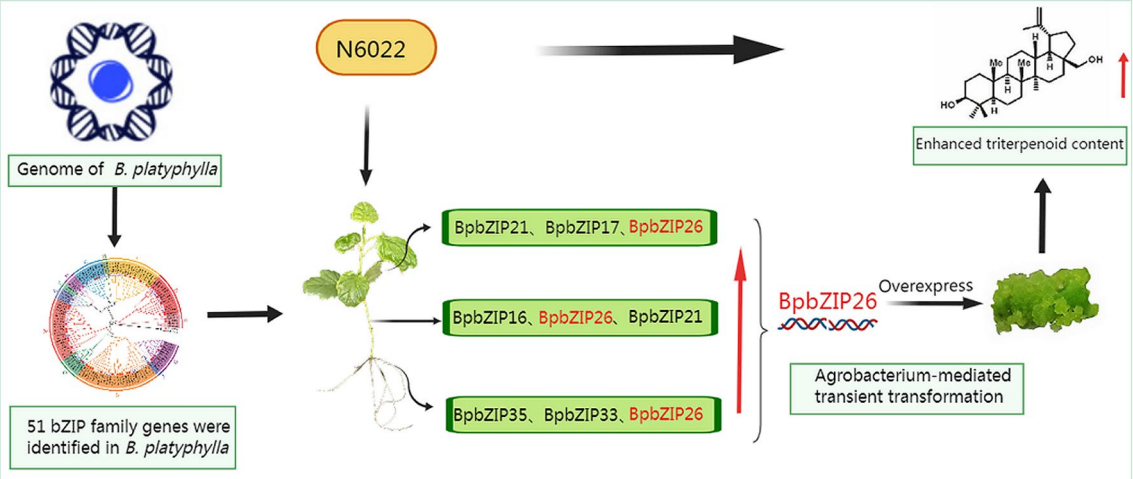
Keywords: bZIP, Gene family, Triterpenoid, S-nitrosothiol treatment, *Betula platyphylla*

†Bo Wang and Haixin Yang have contributed equally to this work

*Correspondence: gzf325@126.com

Key Laboratory of Saline-Alkali Vegetation Ecology Restoration, Ministry of Education, Northeast Forestry University, Harbin 150040, China

Graphical Abstract



Introduction

Basic leucine zipper (bZIP) is one of the largest transcription factors (TFs) families in plants, its proteins contain a highly conserved 60–80 amino acid sequence that includes a basic region (N-×7-R/K sequence) and a leucine zipper [1, 2]. Increasing number of bZIP have recently been identified in multiple plant species based on the whole genome or full-length transcriptome sequences, and its family size are different, such as 78 in *A. thaliana* [1], 47 in *Betula pendula Roth* [3], 125 in *Zea mays* [4]. Based on conserved domains and sequence similarities of their basic regions, bZIP TFs can be divided into 11–14 subgroups that have different functions [1, 3–6]. For example, bZIP family of *A. thaliana* was divided into 13 groups, most of Group A bZIPs involved in ABA signaling, Group F bZIPs regulated Zn transporters, Group H and G subfamilies regulated photoresponse [1]. Growing studies suggest that bZIP TFs are crucial in plant growth, development, regulation of secondary metabolite synthesis, and response to environmental stress [7, 8]. However, there is still a large proportion of bZIP TFs has not been identified their function in plants.

Nitric oxide (NO) is a signaling molecule distributing throughout all living organisms, and it is involved in multiple plant processes, including growth, development, and biotic and abiotic stress responses [9, 10]. The accumulating data indicate that NO is executed through S-nitrosylation, which is the addition of an NO moiety to a protein cysteine thiol to form an S-nitrosothiol (SNO) [10]. Similar to other posttranslational modifications, S-nitrosylation regulates protein activities, including stability, biochemical activity, subcellular localization, and protein–protein interaction. The difference is that S-nitrosylation can be reversed by S-nitrosoglutathione reductase (GSNOR), it degrades S-nitrosoglutathione (the major cellular NO donor) to oxidized glutathione (GSSG) and ammonia. Plants with null or reduced expression of GSNOR show increased levels of total SNO, and conversely, that GSNOR overexpressing plants show reduced SNO content [10, 11]. Therefore, the level of S-nitrosated proteins corresponds to SNO levels.

Our precious study verified that SNO enhanced betulin production using *BpGSNOR* transgenic *B. platyphylla* seedlings by RNAi silencing and GSNOR inhibitor N6022, and it indicated that protein S-nitrosylation mediated betulin production in *B. platyphylla*, which is a pioneer hardwood tree species with rich medical triterpenoid [12, 13]. Some bZIP TFs can promote secondary metabolite synthesis, such as *VvbZIP36, PybZIP,* and *PgbZIP* promotes anthocyanin accumulation in grapevine, Pear, and pomegranate, respectively [11, 14, 15]. Given the importance of bZIP TFs in secondary metabolite biosynthesis in plants, the functional characterization of bZIP TFs family members in betulin biosynthesis of *B. platyphylla* has not been systematically investigated, especially under SNO treatment. Therefore, in this study, the latest *B. platyphylla* reference genome was used to characterize bZIP TFs [16], a bZIP gene predicted to be involved in triterpenoid synthesis, was cloned and verified via transient transformation in *B. platyphylla* under SNO treatment. The results of this study will contribute to the functional characterization of bZIP TFs in *B. platyphylla.*

Materials and methods

Identification of BpbZIP genes

All annotated sequences were obtained from the genome of *B. platyphylla* (accession code PRJNA285437) [16], and bZIP domain (PF00170) downloaded from the Pfam database was used to detect the possible bZIP protein of *B. platyphylla* through the HMM search program (*E* value $< 1 \times 10^{-5}$). All putative proteins were subjected to conserved structural domain identification using SMART software and the NCBI–CDD database [17]. Fifty-one *BpbZIP* family genes were identified and numbered according to their positions on chromosomes.

Analysis of physiochemical properties, localization, and gene structures

The physicochemical properties, protein molecular weights (MW), and theoretical isoelectric points (pI) of the 51 identified bZIP proteins were analyzed by ExPASy-ProtParam. The subcellular localization was predicted by Plant-mPLoc [17]. The chromosome location information was analyzed using TBtools software. The conserved motifs of the 51 BpbZIP proteins were analyzed by Multiple expectation maximization for motif elicitation (MEME), and the maximum number of motifs was set to 10 (width range of motif = 6–300 residues). PHYLOGENY in MEGA X software was adopted to construct a maximum-parsimony tree through 1000 bootstrap replications. The above precited results were visualized using TBtools software [17, 18].

Construction of the phylogenetic trees

Clustal W in MEGA X software were used to align the amino acid sequences of the 51 BpbZIP proteins and 78 *A. thaliana* bZIP proteins, and PHYLOGENY in MEGA X software was used to draw a neighbor-joining tree (1000 bootstrap replications) [4, 17]. The phylogenetic tree was modified using the online software EvolView.

Cis-acting element predictions and evolution analysis

The 2000 bp upstream sequence of each *B. platyphylla* bZIP was extracted and submitted to PlantCARE for cis-regulating elements functions prediction analysis. The duplication events in *BpbZIPs*, and syntenic relationships among *B. platyphylla*, *A. thaliana*, *G. max*, and *P. trichocarpa* were analyzed using TBtools software [17, 18]. The above precited results were visualized using TBtools software.

Plant materials and N6022 treatment

Thirty-day-old seedlings of *B. platyphylla* obtained from sterile seeds were treated with 60 μmol L^{-1} 3-(5-(4-(1*H*-imidazol-1-yl) phenyl)-1-(4-carbamoyl-2-methylphenyl)-1*H*-pyrrol-2-yl) propionic acid (N6022, a GSNOR inhibitor) for 24 h, and 15-day-old calli of *B. platyphylla* obtained from tissue-cultured seedlings were used for *Agrobacterium*-mediated transient transformation and N6022treatment. The controls were treated with the same volume of distilled water. N6022 was purchased from Sigma Corporation (St Louis, MO, USA). The seedlings were planted in a woody plant medium supplemented with 20 g L^{-1} of sucrose. The calli were cultured in B_5 medium supplemented with 0.3 mg L^{-1} of 6-benzyladenine, 0.6 mg L^{-1} of thidiazuron, and 20 g L^{-1} of sucrose. The pH of the medium was adjusted to 5.6 ± 0.2 prior to autoclaving [19]. Fresh samples frozen with liquid nitrogen were used for gene expression, and samples dried through the oven-drying method were used for the analysis of triterpenoid content [19].

Cloning of full-length *BpbZIP26*

The full-length sequence of *BpbZIP26* was amplified by the following PCR primers: F: ATATTGTCAACACAT TGCCTG, R: AAACAAAATGATCTTACGCTT. PCR amplification was set as follows: 94 °C for 5 min; 35 cycles of 98 °C for 10 s, 50 °C for 45 s, and 72 ° C for 1 min; and 72 °C for 10 min. Positive colonies (purified PCR amplification fragment ligated with pMD^{TM}18-T vector) were sequenced at Rui Biotech (Beijing) [17].

Agrobacterium-mediated transient transformation

Agrobacterium tumefaciens strain LBA4404 harboring pBWA(V)HS-BpbZIP26-GLosgfp (overexpression vector) or pBWA(V)KS-BpbZIP26-GUS (RNAi vector) was used to infect 15-day-old *B. platyphylla* calli (soaked in 25% sucrose for 5 min) for 1 h. The infection solution was reported by Liu et al. [16], and the main reagent as follows: 2 mM L^{-1} of MES–KOH (pH 5.4), 10 mmol L^{-1} of $CaCl_2$, 120 μmol L^{-1} of acetosyringone (AS), 2% sucrose, 270 mmol L^{-1} of mannitol, and 200 mg L^{-1} of dithiothreitol + 0.02% Tween. The infected calli (15 g calli per replicate) were cultured in B_5 liquid medium containing 100 μmol L^{-1} of AS for 2 days in the dark at 28 °C [17]. Then, the infected calli were washed with distilled water for analysis of gene expression and total triterpenoid content.

Determination of the total triterpenoid content

Fried samples (0.05 g) were accurately weighed and soaked in 5 mL of 95% ethanol for 24 h. The samples were extracted at 70 °C for 1 h in a water bath, and then ultrasound-assisted extraction (10 kHz) for 40 min. After centrifugation at 4000 rmp for 10 min, 1 mL of the supernatant solution was obtained for content analysis [19]. The total triterpenoid content was determined using the vanillin–glacial acetic acid colorimetric method with betulin as the standard, and the linear equation was

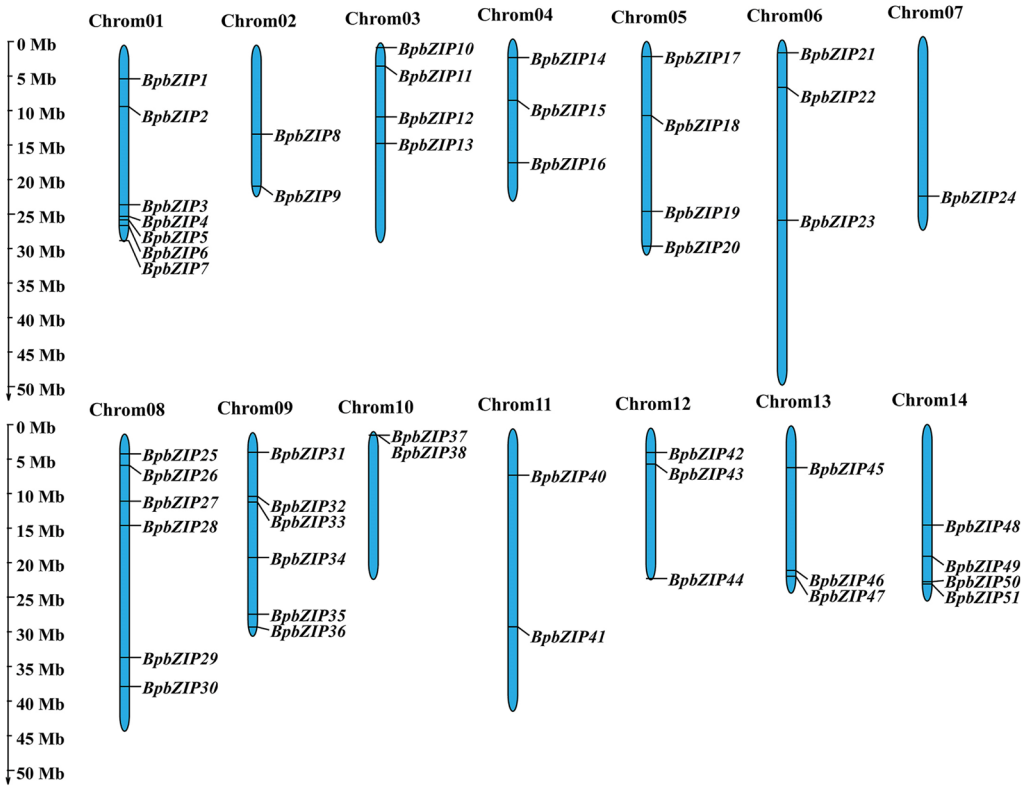


Fig. 1 Chromosomal locations of *BpbZIP*s in *B. platyphylla*. The chromosomal position of 51 *BpbZIP* family genes were mapped according to the *B. platyphylla* genome (PRJNA285437) using TBtools software. The left scale indicated the length of the chromosome

$y = 0.03483x + 0.0002$ ($R^2 = 0.9995$), where x indicates the absorbance of the solution at 551 nm.

Gene expression analysis

The total RNA was isolated using a CTAB-based method, and 1 μg RNA of each sample was reversed into cDNA according to instructions of the PrimeScriptTM RT reagent Kit (TaKaRa, Japan). The Taqman probes and primers are presented in Additional file 1: Tables 1, 2. PCR amplification was performed on a Roche LightCycler 480 real-time PCR system as follows: 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Each RT-qPCR analysis was performed with three technical replicates. Gene expression data were calculated with the $2^{-\Delta\Delta Ct}$ method [20].

Statistical analysis

The data presented in the figures were the mean ± standard deviation of three biological replicates, and analyzed through one-way ANOVA using SPSS version 21.0. The different letters show significant differences among means ($P < 0.05$, Tukey's test) [17].

Results

Genome-wide identification of *BpbZIPs*

Fifty-one genes with conserved bZIP domains were identified in the *B. platyphylla* genome and named *BpbZIP1–BpbZIP51* sequentially according to their positions on chromosomes. All *BpbZIP* genes were unevenly distributed on 14 chromosomes, with chromosome 1 containing the most genes (7 *BpbZIP* genes) and chromosomes 7 containing a single *BpbZIP* gene, and most of *BpbZIPs* were distributed at both ends of the chromosomes (Fig. 1).

The physicochemical properties of 51 BpbZIP proteins showed that the number of amino acids ranged from138 (BpbZIP25 and BpbZIP30) to 584 (BpbZIP17), molecular weight ranged from 15.71 (BpbZIP25 and BpbZIP30) to 63.67 (BpbZIP17) kD, and isoelectric points ranged from 4.50 (BpbZIP22) to 10.16 (BpbZIP7) (Table 1). The subcellular location predicted that most of the proteins were nuclear proteins. Only BpbZIP2, BpbZIP7, BpbZIP22, BpbZIP29, BpbZIP40, and BpbZIP48 were distributed in the chloroplast.

Table 1 Sequence information of *BpbZIP* gene family

Name	Sequence ID	Amino acid number	Molecular weight (kD)	Theoretical pI	Subcellular localization
BpbZIP1	>BPChr07G09851	199	23.08	5.55	Nuclear
BpbZIP2	>BPChr01G24961	198	21.48	10.08	Chloroplast
BpbZIP3	>BPChr01G05288	435	46.62	8.54	Nuclear
BpbZIP4	>BPChr01G18070	145	16.83	7.84	Nuclear
BpbZIP5	>BPChr01G22783	155	17.83	7.88	Nuclear
BpbZIP6	>BPChr01G22848	484	52.71	6.18	Nuclear
BpbZIP7	>BPChr01G16976	278	30.74	10.16	Chloroplast
BpbZIP8	>BPChr01G31933	304	33.65	4.82	Nuclear
BpbZIP9	>BPChr05G14456	145	16.35	6.84	Nuclear
BpbZIP10	>BPChr05G17438	380	40.47	6.42	Nuclear
BpbZIP11	>BPChr05G31486	446	48.42	6.69	Nuclear
BpbZIP12	>BPChr05G17931	159	18.08	5.90	Nuclear
BpbZIP13	>BPChr06G02197	339	35.83	5.33	Nuclear
BpbZIP14	>BPChr06G29443	433	46.17	6.86	Nuclear
BpbZIP15	>BPChr06G22245	263	29.17	6.45	Nuclear
BpbZIP16	>BPChr02G14600	214	23.28	9.34	Nuclear
BpbZIP17	>BPChr02G10510	584	63.67	6.41	Nuclear
BpbZIP18	>BPChr13G21953	363	40.84	4.91	Nuclear
BpbZIP19	>BPChr13G00887	488	54.02	5.92	Nuclear
BpbZIP20	>BPChr13G00422	264	29.33	5.73	Nuclear
BpbZIP21	>BPChr04G23855	159	18.08	5.90	Nuclear
BpbZIP22	>BPChr04G04435	506	55.47	4.50	Chloroplast
BpbZIP23	>BPChr04G00610	332	37.22	6.62	Nuclear
BpbZIP24	>BPChr03G00747	378	41.94	9.80	Nuclear
BpbZIP25	>BPChr03G10962	138	15.71	10.05	Nuclear
BpbZIP26	>BPChr03G13918	191	21.76	7.94	Nuclear
BpbZIP27	>BPChr03G02600	335	36.87	8.02	Nuclear
BpbZIP28	>BPChr08G15472	280	31.69	6.65	Nuclear
BpbZIP29	>BPChr08G15858	306	34.50	5.84	Chloroplast
BpbZIP30	>BPChr08G07606	138	15.71	10.05	Nuclear
BpbZIP31	>BPChr08G05419	420	45.82	6.22	Nuclear
BpbZIP32	>BPChr08G16217	170	19.17	6.84	Nuclear
BpbZIP33	>BPChr08G11307	184	21.25	6.16	Nuclear
BpbZIP34	>BPChr14G12548	471	51.76	7.83	Nuclear
BpbZIP35	>BPChr14G12853	503	56.83	7.17	Nuclear
BpbZIP36	>BPChr14G27906	254	27.41	9.56	Nuclear
BpbZIP37	>BPChr14G27794	450	50.04	7.98	Nuclear
BpbZIP38	>BPChr09G20109	328	35.89	9.06	Nuclear
BpbZIP39	>BPChr09G29729	452	49.87	6.22	Nuclear
BpbZIP40	>BPChr09G29789	186	21.25	9.54	Chloroplast
BpbZIP41	>BPChr09G12275	268	29.58	6.94	Nuclear
BpbZIP42	>BPChr09G16384	336	37.47	6.03	Nuclear
BpbZIP43	>BPChr09G20563	156	17.44	8.89	Nuclear
BpbZIP44	>BPChr11G18682	428	48.18	9.06	Nuclear
BpbZIP45	>BPChr11G07209	319	35.92	6.19	Nuclear
BpbZIP46	>BPChr11G17764	381	40.90	6.27	Nuclear
BpbZIP47	>BPChr12G25846	413	43.87	6.25	Nuclear
BpbZIP48	>BPChr12G08218	289	32.50	6.70	Chloroplast
BpbZIP49	>BPChr12G25179	317	35.55	6.18	Nuclear

Table 1 (continued)

Name	Sequence ID	Amino acid number	Molecular weight (kD)	Theoretical pI	Subcellular localization
BpbZIP50	>BPChr10G21732	304	33.84	9.66	Nuclear
BpbZIP51	>BPChr10G21739	222	25.58	6.66	Nuclear

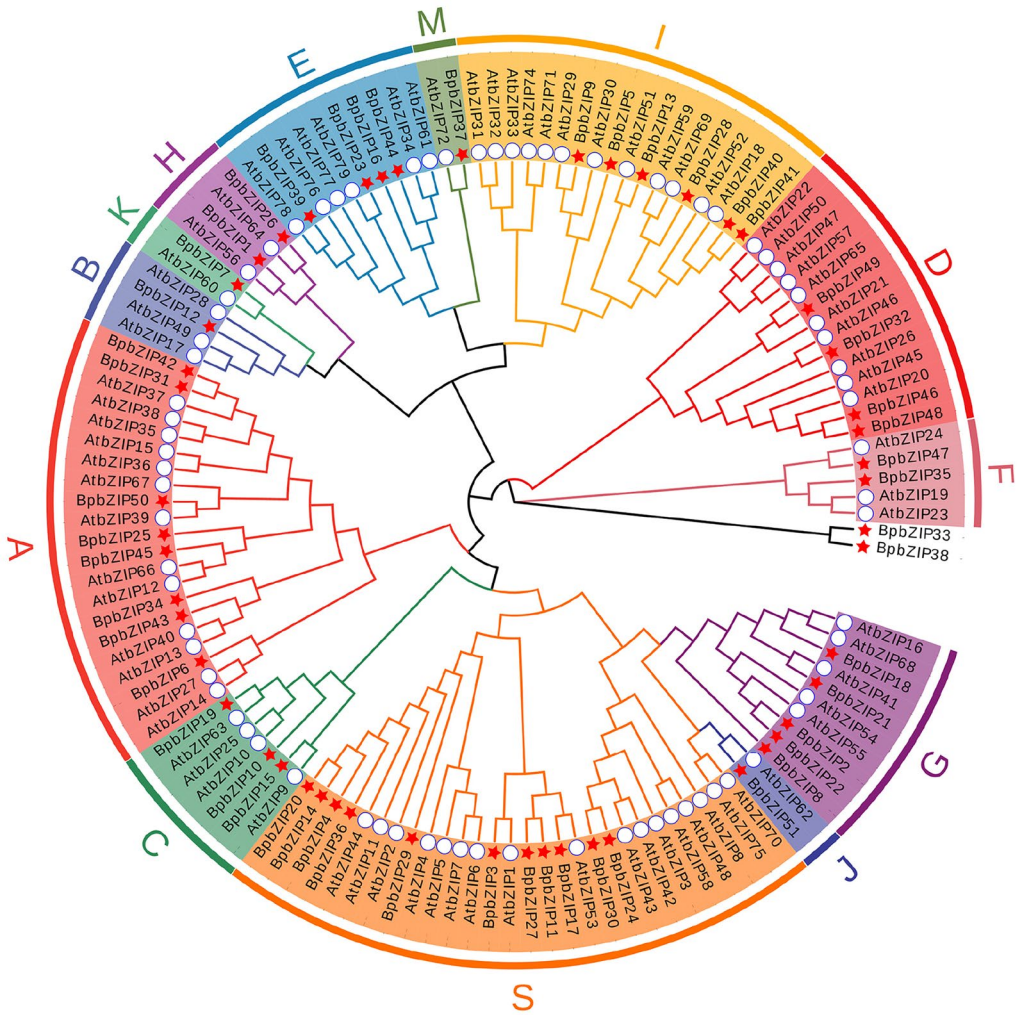


Fig. 2 Phylogenetic tree of *B. platyphylla* and *A. thaliana*. The outermost letters A to S indicated that 51 BpbZIP proteins were divided into 13 subgroups except for BpbZIP33 and BpbZIP38 according to the classification of *A. thaliana* bZIP proteins, white dots were AtbZIP proteins, and red star were BpbZIP proteins

Phylogenetic analysis of BpbZIPs

In accordance with the classification of *A. thaliana* bZIP proteins, 51 BpbZIP proteins were divided into 13 subgroups except for BpbZIP33 and BpbZIP38, which were named as Subgroups A, B, C, D, E, F, G, H, I, J, K, M, and S (Fig. 2). The largest subgroup S contained 11 BpbZIP members, followed by the Groups A and I containing 8 and 6 BpbZIP members, respectively. Subgroup J, K, and M all had only one protein. This suggested the members of bZIP proteins involved in plant starvation signalling, abscisic acid or stress signalling and vascular development, etc. [1].

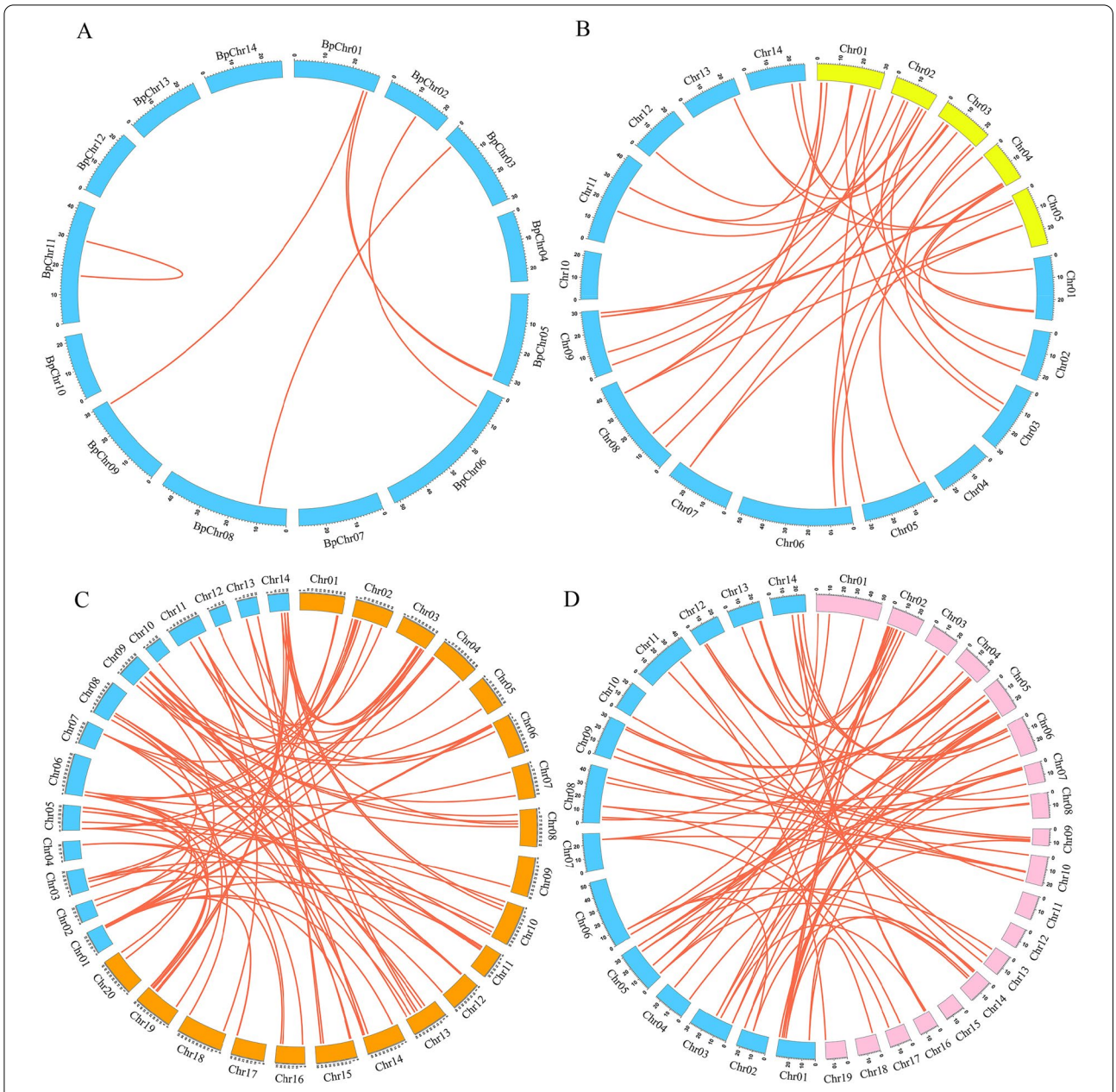


Fig. 3 Evolutionary analysis of *BpbZIPs*. (**a**) 12 duplication events were detected in the *B. platyphylla* genome. (**b**) Synteny analysis of *BpbZIP* genes between *B. platyphylla* and *A. thaliana*. (**c**) Synteny analysis of *BpbZIP* genes between *B. platyphylla* and *G. max*. (**d**) Synteny analysis of *BpbZIP* genes between *B. platyphylla* and *P. trichocarpa*

Evolutionary analysis of BpbZIPs

As shown in Fig. 3a, 12 duplication events with 51 *BpbZIP* genes were detected in the *B. platyphylla* genome. One pair of genes (*BpbZIP40* and *BpbZIP41*) was found to have undergone a tandem duplication event and five pairs of genes (*BpbZIP4* and *BpbZIP36*, *BpbZIP4* and *BpbZIP20*, *BpbZIP*6 and *BpbZIP20*, *BpbZIP8* and *BpbZIP22*, *BpbZIP11* and *BpbZIP27*) underwent a fragment duplication event. This evidence suggested that fragment duplication events were a major driver of *BpbZIP* gene diversity. The syntenic relationships of the *BpbZIP* genes showed that 28 orthologs existed between *B. platyphylla* and *A. thaliana* (Fig. 3b), 83 orthologs existed between *B. platyphylla* and *G. max* (Fig. 3c), and 73 orthologs existed between *B. platyphylla* and *P. trichocarpa* (Fig. 3d). The differences in numbers of

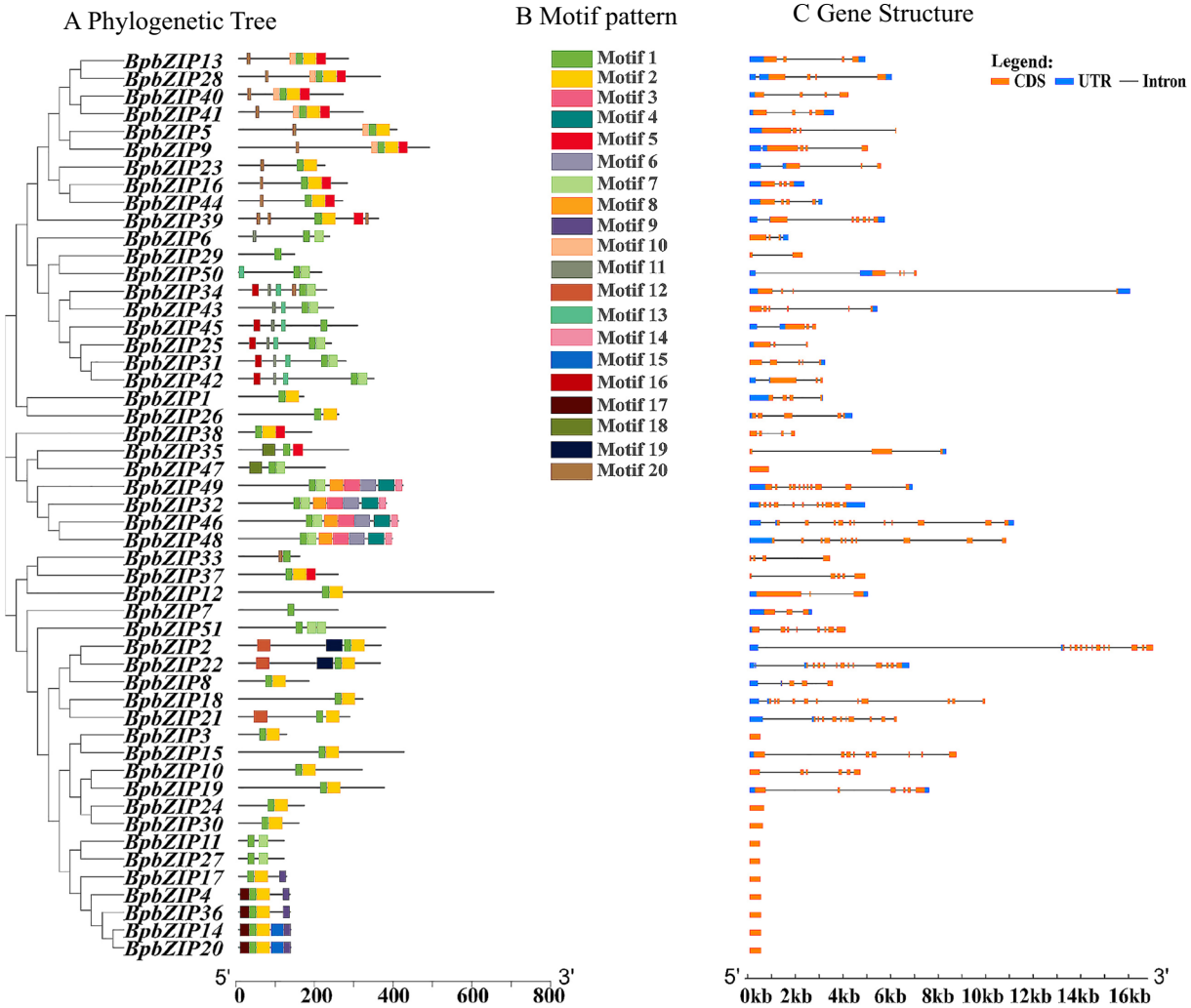


Fig. 4 Gene structures of the *BpbZIP* genes. (**a**) The phylogenetic tree of 51 BpbZIP proteins. (**b**) 20 motifs in the BpbZIPs were represented by different colored boxes. (**c**) Exon/intron structures of *BpbZIP* genes. Yellow boxes, blue boxes, and gray line indicated exons, untranslated regions (UTR), and introns at each *BpbZIP* gene, respectively

orthologous pairs were related to the evolutionary distance [21].

Conserved motifs and gene structures

A total of 20 conserved amino acid motifs were identified in the BpbZIP proteins (Fig. 4b), and motifs with similar structures and domains were clustered into one group indicating that they had an analogous function (Fig. 4a), such as group I (BpbZIP13, BpbZIP28, BpbZIP40, BpbZIP41, BpbZIP5, and BpbZIP9). Motif1 was distributed in all members of the BpbZIPs, which was recognized as bZIP conserved domain sequence. However, some motifs were very rare, such as motif 19 only found in group I, motif 18 in group F.

Gene structure is one of the important parameters for gene family evolution that further supports phylogenetic trees. Gene structure analysis was performed on the 51 BpbZIP genes (Fig. 4c). The results showed that 78.43% (40/51) of the *BpbZIPs* had introns varying from 1 (*BpbZIP29*) to 13 (*BpbZIP2*, *46*), and the eleven intronless genes were *BpbZIP3*, *4*, *11*, *14*, *17*, *20*, *24*, *27*, *30*, *36*, and *47*. In addition, 70.59% (36/51) of the *BpbZIPs* had untranslated regions (UTRs) varying from 1 (*BpbZIP1*, *5*, *6*, *15*, *25*, *31*, *35*, *40*, *43*, *48*) to 3 (*BpbZIP22*, *46*), and the 15 genes (*BpbZIP3*, *4*, *11*, *14*, *17*, *20*, *24*, *27*, *29*, *30*, *33*, *36*, *37*, *38*, *47*) had no UTR.

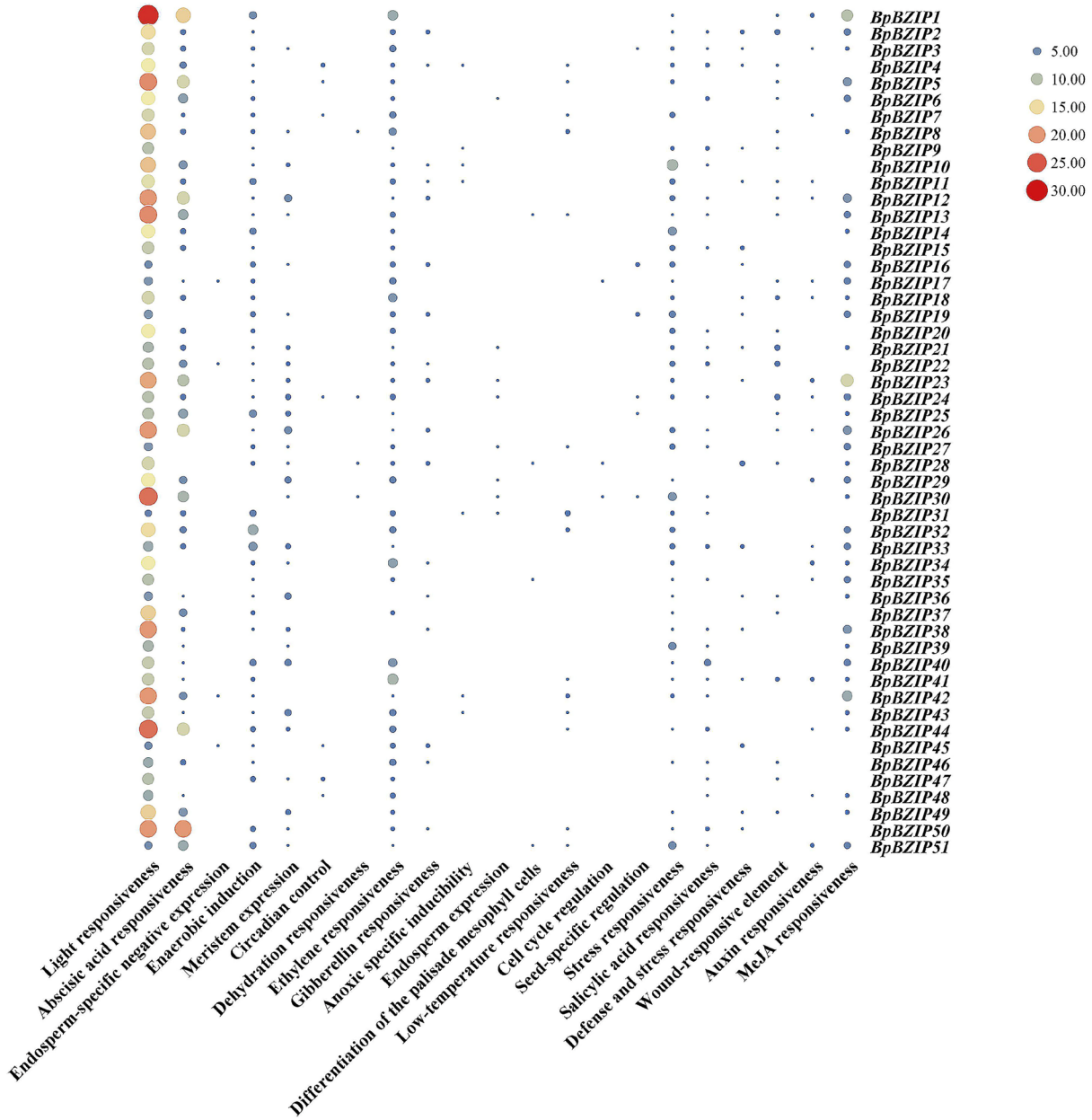


Fig. 5 Cis-elements in the promoter regions of *BpbZIPs*. The 2.0 kb promoter region located upstream of each *BpbZIP* gene was used to predict by PlantCARE. The number of different kinds of cis-elements were represented with the colored circle

The cis-elements in the promoter regions of *BpbZIP*s

The 2.0 kb promoter region located upstream of the transcriptional start site of each *BpbZIP* gene was used to predict their possible expression regulation patterns (Fig. 5). The cis-elements of the *BpbZIP*s belonging to the same group in phylogenetic analysis did not show the same pattern. The number of responsive elements ranged from 25 (*BpbZIP26*) to 84 (*BpbZIP1*), and light, hormone, and stress were three main categories of responsive elements. Light responsive elements were the most prevalent in all the *BpbZIP*s promoters varying from 4(*BpbZIP36*, *47*) to 34(*BpbZIP1*), and Gbox and box4 were the top two responsive elements. ABA and JA were the dominant hormone elements. Wound-responsive element was rich in the stress responsive elements. The different types and amounts of cis-elements in the promoters of *BpbZIPs* suggested that they might had different functions in *B. platyphylla* growth and development.

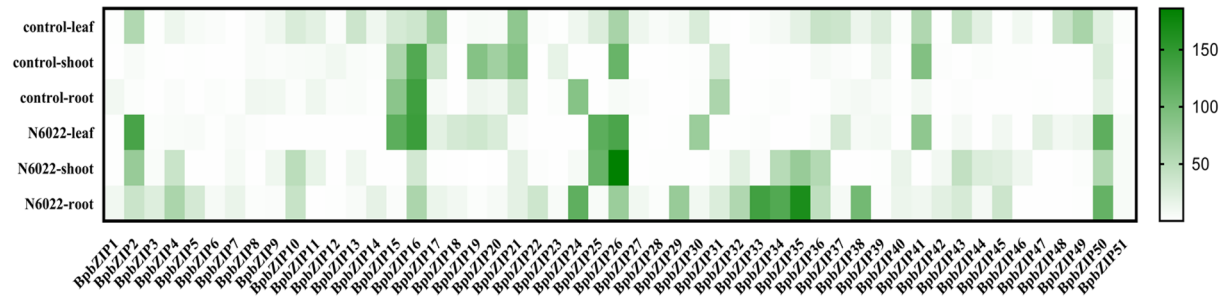


Fig. 6 Gene expression levels of *BpbZIP*s in seedlings of *B. platyphylla* under N6022 treatment. Thirty-day-old seedlings of *B. platyphylla* obtained from sterile seeds were treated with 60 μmol L^{-1} 3-(5-(4-(1*H*-imidazol-1-yl) phenyl)-1-(4-carbamoyl-2-methylphenyl)-1*H*-pyrrol-2-yl) propionic acid (N6022, a GSNOR inhibitor) for 24 h

Gene expression of BpbZIPs under S-nitrosation treatment

qRT-PCR was used to investigate gene expression patterns of *BpbZIPs* in response N6022 treatment. The results revealed that all *BpbZIP* genes expressed in control tissues of *B. platyphylla*, and 62.7% *BpbZIPs* (32 genes) in leaves were higher than that of stems and roots (Fig. 6). Among them, *BpbZIP21*, *BpbZIP17* and *BpbZIP26* highly expressed in leaves, *BpbZIP16*, *BpbZIP26* and *BpbZIP21* in stems, *BpbZIP16*, *BpbZIP24* and *BpbZIP15* in roots. N6022 treatment changed gene expression levels of *BpbZIPs* in *B. platyphylla* seedlings, *BpbZIP16*, *BpbZIP2* and *BpbZIP26* highly expressed in leaves, and their increases were 3.96, 2.36, and 2.06 times greater than those of controls, respectively. *BpbZIP 25* and *BpbZIP26* highly expressed in stems under N6022 treatment, and their increases were 19.53 and 1.70 folds higher than those of controls, respectively. *BpbZIP35*, *BpbZIP33,* and *BpbZIP26* highly expressed in roots under N6022 treatment, their increases were 27.4, 15.8, and 12.50 folds greater than those of controls, respectively. The above results suggested that *BpbZIP26* may play a key role in *B. platyphylla* responses to S-nitrosation treatment. Hence, we cloned *BpbZIP26* via PCR (Additional file 1: Figs. 1, 2).

Overexpression of *BpbZIP26* enhanced triterpenoid production under S-nitrosation treatment

Our previous study showed that N6022 treatment significantly enhanced triterpenoid content [11, 12], and it also significantly increased gene expression of *BpbZIP26*. To investigate the function of *BpbZIP26* in triterpenoid synthesis, overexpression and silencing vector of *BpbZIP26* were constructed to transfer into *B. platyphylla* calli. After 3 days of *Agrobacterium*-mediated transient transformation, the silencing of *BpbZIP26* in *B. platyphylla* calli (0.44 times than that of untransformed calli) significantly decreased the triterpenoid contents (40.46%) and reduced the gene expression of *BpCAS*, *BpLUS*, and *BpβAS*, which are key enzyme genes for triterpenoid synthesis. The overexpression of *BpbZIP26* in *B. platyphylla* calli (812 times than that of untransformed calli) enhanced the triterpenoid contents (26.21%) and increased the gene expression of *BpCAS*, *BpLUS*, and *BpβAS*. N6022 treatment further enhanced triterpenoid contents (90.38% and 65.44%) and gene expression of *BpCAS*, *BpLUS*, and *BpβAS* in control and overexpression of *BpbZIP26* calli of *B. platyphylla* (Fig. 7). The above results suggested that *BpbZIP26* mediated triterpenoid production under control and S-nitrosation treatment.

Discussion

In our study, 51 *BpbZIP*s were identified in the *B. platyphylla* genomes for the first time, and its family size was larger than that the same genus of *B. pendula* (47) [3]. Further analysis showed that the genome size of *B. platyphylla* (441 Mb) and *B. pendula* (440 Mb) was the about the same size, but *bZIP*s of *B. pendula* and *B. platyphylla* were divided into 10 and 13 groups according to the clustering with *A. thaliana*, respectively. In addition, they all had 1–2 genes that are not clustered with *A. thaliana*. It can be seen that there are differences in bZIP gene family size and clustering among Betula species, different clustering suggested that they could have different function, those will be verified experimentally in the future.

Genomic chromosome localization analysis showed that *bZIP*s of *Lycopersicon esculentum* (69 *SlbZIPs*) and *G. max* (160 *GmbZIPs*) were unevenly distributed in all chromosomes, but *bZIP*s in *Vitis vinifera* (55 *VvbZIPs*), *Citrullus lanatus* (62*ClabZIP*), and *Nicotiana tabacum* (77 *NtbZIP*s) were concentrated in some chromosomes. In addition, 6 *bZIP*s in *Raphanus sativus* (135 *RsbZIPs*) and 9 genes in *Pyrus breschneideri* (92 *PbrbZIPs*) had unassigned scaffolds in chromosomes [22–28]. In our study, 51 *BpbZIP*s were unevenly distributed in all the chromosomes of *B. platyphylla*. The above

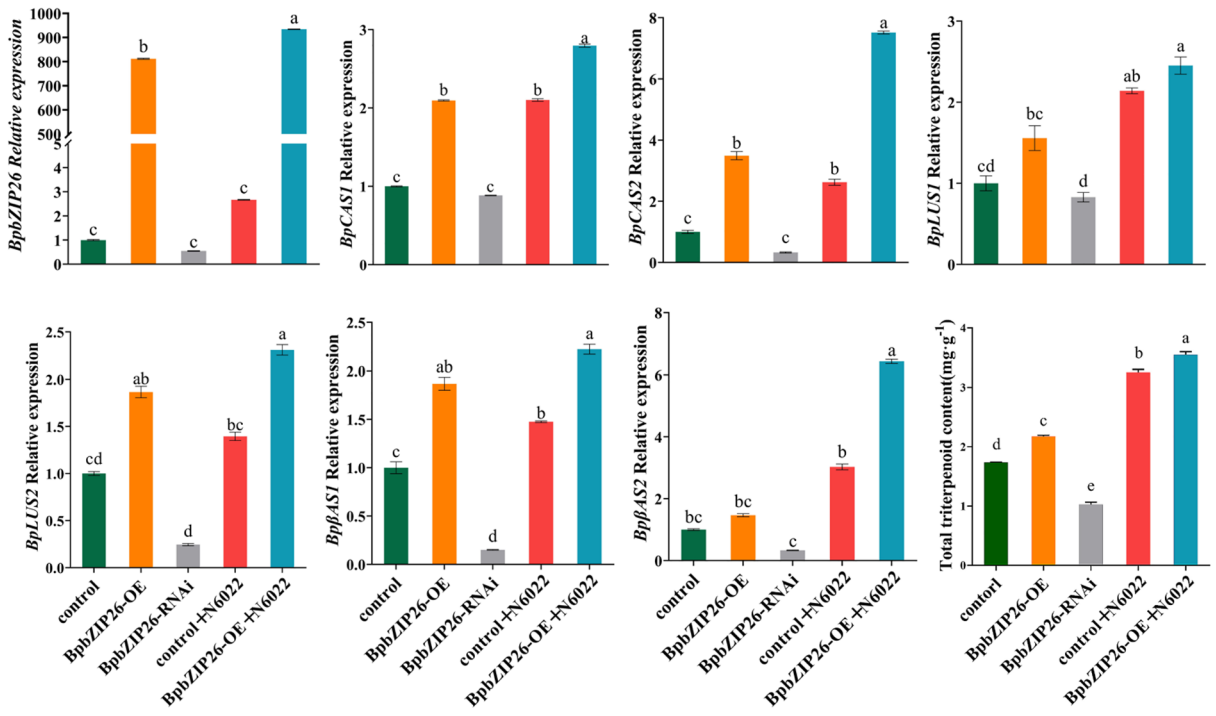


Fig. 7 *BpbZIP 26* regulated triterpenoid production using *Agrobacterium*-mediated transient transformation. Fifteen-day-old calli of *B. platyphylla* were used for *Agrobacterium*-mediated transient transformation and 60 μmol L^{-1} N6022 treatment

results revealed that the number of *bZIP* gene family was irrelevant to the chromosome size. Evolutionary analysis of *BpbZIPs* showed that 28 orthologs existed between *B. platyphylla* and *A. thaliana*, 83 orthologs existed between *B. platyphylla* and *G. max*, and 73 orthologs existed between *B. platyphylla* and *P. trichocarpa*. It seems that the evolution events in bZIP gene family members have happened before the divergence of species, which affected their gene family numbers and evolutionary distance.

The cis-elements in the promoter regions of *bZIPs* and increasing experimental data indicated that the bZIP genes played important roles in plant growth and response to multiple stresses [1, 2]. NO is a ubiquitous gasotransmitter produced in living cells under normal as well as under conditions of biotic and abiotic stress. The major bioactivity of NO is executed via S-nitrosylation by covalently adding an NO group onto the reactive cysteine thiol of a protein to form SNO, which is regulated by GSNOR [10, 11]. It can be seen that both *bZIPs* and NO are all involved in plant growth and stress response. Therefore, how *bZIPs* responds to SNO has not yet been reported.

To investigate the effect of SNO on gene expression of 51 *BpbZIPs*, 30-day-old seedlings of *B. platyphylla* obtained from sterile seeds were treated with 60 μmol L^{-1} N6022 (a GSNOR inhibitor) for 24 h. The results revealed that gene expression levels of 62.7% *BpbZIPs* (32 genes) in leaves were higher than that of stems and roots in *B. platyphylla*, and N6022 treatment changed expression levels of most *BpbZIP* genes. Among of them, N6022 treatment significantly increased gene expression of *BpbZIP26* in leaves, stems, and roots of *B. platyphylla*. The above results suggested that the *BpbZIP* genes can respond to SNO, and *BpbZIP26* may play a key role in responses to SNO.

Some bZIP TFs can regulate secondary metabolite production, such as *BcbZIP134* decreased the biosynthesis of saikosaponin, *VvbZIP36* promotes anthocyanin accumulation in grapevins [14]. Our precious study verified that SNO enhanced triterpenoid production using *BpGSNOR* transgenic *B. platyphylla* seedlings by RNAi silencing and GSNOR inhibitor N6022, and SNO also significantly increased gene expression of *BpbZIP26*. To investigate the function of *BpbZIP26* in triterpenoid synthesis, we cloned *BpbZIP26* and tentatively verified *BpbZIP26* mediated triterpenoid production under control and N6022 treatment using *Agrobacterium*-mediated transient transformation, which has been proved by experiment that the results of transient transformation were consistent with that based on stable transformation in *B. platyphylla* [29]. The above result will provide gene

resources for further improving the content of triterpenoid in *B. platyphylla*.

Conclusion

We identified 51 *BpbZIP*s in the *B. platyphylla* through genome-wide study, and divided into 13 subgroups according to the classification of *A. thaliana* bZIP proteins. All *BpbZIP* genes were unevenly distributed on 14 chromosomes, and12 duplication events were detected in the *B. platyphylla* genome. N6022 treatment changed gene expression levels of most *BpbZIPs* in leaves, stems, and roots, and gene expression levels of *BpbZIP26* in all tissues of *B. platyphylla* was significantly enhanced under N6022 treatment. *BpbZIP26* mediated triterpenoid production, and N6022 treatment further enhanced triterpenoid production in *BpbZIP26* overexpression calli of *B. platyphylla* using *Agrobacterium*-mediated transient transformation.

Supplementary Information

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Additional file 1. Table S1. Sequences of primer pairs of housekeeping genes for quantitative real-time RT-PCR assay. **Table S2** Sequences of primer pairs of BpbZIPs for quantitative real-time RT-PCR assay. **Fig. S1** Electrophoresis chart of PCR products of *BpbZIP26* in *B. platyphylla*. **Fig. S2** Amino acid sequences of *BpbZIP26* in *B. platyphylla*.

Author contributions

Gui-Zhi Fan, Huinin-Liu, and Xing-lei Gao wrote the main manuscript text and Bo Wang and Hai-xin Yang prepared Figs. 1–7. All authors reviewed the manuscript.

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Data availability

The data that support the finding of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This manuscript is an original paper and has not been published in other journals. The authors agreed to keep the copyright rule.

Consent for publication

The authors agreed to the publication of the manuscript in this journal.

Competing interests

The authors declare no conflict of interests.

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References

1. Droge-Laser W, Snoek BL, Snel B, Weiste C. The Arabidopsis bZIP transcription factor family an update. Curr Opin Plant Biol. 2018;45:36–49.
2. Wang LF, Zhu JF, Li XM, Wang SM, Wu J. Salt and drought stress and ABA responses related to bZIP genes from *V. radiata* and *V. angularis*. Gene. 2018;651:151–60.
3. Jing YN, Yu Y, Wang HZ, Wu Y, Li CH. Genome-wide identification and expression analysis of the bZIP gene family in silver birch (*Betula pendula* Roth.). J For Res. 2022;33(5):1615.
4. Wei K, Chen J, Wang Y, Chen Y, Chen S, Lin Y, Pan S, Zhong X, Xie D. Genome-wide analysis of bZIP-encoding genes in maize. DNA Res. 2012;19(6):463–76.
5. Fan L, Xu L, Wang Y, Tang M, Liu L. Genome- and transcriptome-wide characterization of bZIP gene family identifies potential members involved in abiotic stress response and anthocyanin biosynthesis in radish (*Raphanus sativus* L.). Int J Mol Sci. 2019;20(24):6334.
6. Zhao K, Chen S, Yao W, Cheng Z, Zhou B, Jiang T. Genome-wide analysis and expression profile of the bZIP gene family in poplar. BMC Plant Biol. 2021;21:122.
7. Sánchez-Vicente I, Fernández-Espinosa M, Lorenzo O. Nitric oxide molecular targets: reprogramming plant development upon stress. J Exp Bot. 2019;70:4441–60.
8. Gupta KJ, Kaladhar VC, Fitzpatrick TB, Fernie AR, Møller IM, Loake GJ. Nitric oxide regulation of plant metabolism. Mol Plant. 2022;15:228–42.
9. Hussain A, Yun BW, Kim JH, Gupta KJ, Hyung NI, Loake GJ. Novel and conserved functions of S-nitrosoglutathione reductase in tomato. J Exp Bot. 2019;70(18):4877–86.
10. Dröge-Laser W, Snoek BL, Snel B, Weiste C. The Arabidopsis bZIP transcription factor family-an update. Curr Opin Plant Biol. 2018;45:36–49.
11. Liu H, Su J, Zhu Y, Yao G, Allan AC, Ampomah-Dwamena C, Shu Q, Lin-Wang K, Zhang S, Wu J. The involvement of PybZIPa in light-induced anthocyanin accumulation via the activation of PyUFGT through binding to tandem G-boxes in its promoter. Hortic Res. 2019;6:134.
12. Ma K, Jiang Y, Yu ZY, Huang YT, Zhan YG, Fan GZ. H_2S-induced NO/SNO positively promotes betulin production in *Betula platyphylla*. Ind Crops Prod. 2019;140(C):111608.
13. Fan GZ, Nie TT, Huang YT, Zhan YG. GSNOR deficiency enhances betulin production in *Betula platyphylla*. Trees. 2018;32(3):847–53.
14. Tu M, Fang J, Zhao R, Liu X, Yin W, Wang Y, Wang X, Wang X, Fang Y. CRISPR/Cas9-mediated mutagenesis of VvbZIP36 promotes anthocyanin accumulation in grapevine (*Vitis vinifera*). Hortic Res. 2022;9:022.
15. Wang S, Zhang X, Li B, Zhao X, Shen Y, Yuan Z. Genome-wide identification and characterization of bZIP gene family and cloning of candidate genes for anthocyanin biosynthesis in pomegranate (*Punica granatum*). BMC Plant Biol. 2022;22(1):170.
16. Chen S, Wang YC, Yu LL, Zheng T, Wang S, Yue Z, Jiang J, Kumari S, Zheng CF, Tang HB, Li J, Li YQ, Chen JJ, Zhang WB, Kuang HH, Robertson JS, Zhao PX, Li HY, Shu SQ, Yordanov YS, Huang HJ, Goodstein DM, Gai Y, Qi Q, Min JM, Xu CY, Wang SB, Qu GZ, Paterson AH, Sankoff D, Wei HR, Liu GF, Yang CP. Genome sequence and evolution of *Betula platyphylla*. Hortic Res. 2021;8:37.
17. Liu HM, Yu ZY, Fan GZ, Zheng BJ. Genome-level analysis of BpR2R3-MYB family genes transcribed in seedlings of *Betula platyphylla* and BpR2R3-MYB15 enhanced flavonoid production. Chem Biol Technol Agric. 2022;9:37.
18. Chen CJ, Chen H, Zhang Y, Thomas HR, Frank MH, He YH, Xia R. TBtools: an integrative toolkit developed for interactive analyses of big biological data. Mol Plant. 2020;13:1194–202.
19. Jiang Y, Sun FF, Zhan YG, Fan GZ. Effect of carbon monoxide on growth and triterpenoid production in *Betula platyphylla* suspension cells. Chin Tradit Herbal Drugs. 2019;50(15):3681–6.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 2001;25:402–8.
21. Yang XY, Li J, Guo T, Guo B, An XM. Comprehensive analysis of the R2R3-MYB transcription factor gene family in *populus trichocarpa*. Ind Crops Prod. 2021;168: 113614.
22. Liu JY, Chen NN, Chen F, Cai B, Dal Santo SD, Tornielli GB, Pezzotti M, Cheng ZM. Genome-wide analysis and expression profile of the bZIP transcription factor gene family in grapevine (*Vitis vinifera*). BMC Genomics. 2014;15:281.

23. Yang YX, Li JW, Li H, Yang YG, Guang YL, Zhou Y. The bZIP gene family in watermelon: genome-wide identification and expression analysis under cold stress and root-knot nematode infection. Peer J. 2019;7: e7878.
24. Duan LL, Mo ZJ, Fan Y, Li KY, Yang MF, Li DC, Ke YZ, Zhang Q, Wang FY, Fan Y, Liu RX. Genome-wide identification and expression analysis of the bZIP transcription factor family genes in response to abiotic stress in *Nicotiana tabacum L*. BMC Genomics. 2022;23:318.
25. Fan Lx, Xu L, Wang Y, Tang MJ, Liu LW. Genome- and transcriptome-wide characterization of bZIP gene family identifies potential members involved in abiotic stress response and anthocyanin biosynthesis in radish (*Raphanus sativus L.*). Int J Mol Sci. 2019;20:6334.
26. Ma M, Chen QM, Dong HZ, Zhang SL, Huang XS. Genome-wide identification and expression analysis of the bZIP transcription factors, and functional analysis in response to drought and cold stresses in pear (*Pyrus breschneideri*). BMC Plant Biol. 2021;21:583.
27. Li DY, Fu FY, Zhang HJ, Song FM. Genome-wide systematic characterization of the bZIP transcriptional factor family in tomato (*Solanum lycopersicum L.*). BMC Genomics. 2015;16:771.
28. Zhang M, Liu YH, Shi H, Guo ML, Chai MN, He Q, Yan MK, Cao D, Zhao LH, Cai HY, Qin Y. Evolutionary and expression analyses of soybean basic Leucine zipper transcription factor family. BMC Genomics. 2018;19:159.
29. Jia YQ, Niu YN, Zhao HM, Wang ZB, Gao CQ, Wang C, Chen S, Wang YC. Hierarchical transcription factor and regulatory network for drought response in Betula platyphylla. Hortic Res. 2022;9:040.

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