RESEARCH ARTICLE

Characterization of *Brassinazole resistant (BZR)* gene family and stress induced expression in *Eucalyptus grandis*

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Abstract Brassinosteroids (BRs) are a group of plant hormones which play a pivotal role in modulating cell elongation, stress responses, vascular differentiation and senescence. In response to BRs, BRASSINAZOLE-RESISTANT (BZR) transcription factors (TFs) accumulate in the nucleus, where they modulate thousands of target genes and coordinate many biological processes, especially in regulating defense against biotic and abiotic stresses. In this study, 6 BZR TFs of *Eucalyptus grandis* (*EgrBZR*) from a genome-wide survey were characterized by sequence analysis and expression profiling against several abiotic stresses. The results showed that BZR gene family in *Eucalyptus* was slightly smaller compared to *Populus* and *Arabidopsis*, but all phylogenetic groups were represented. Various systematic in silico analysis of these TFs validated the basic properties of BZR, whereas comparative studies showed a high degree of similarity with recognized BZR of other plant species. In the organ-specific expression analyses, 4 *EgrBZR*s were expressed in vascular tissue indicating their possible functions in wood formation. Meanwhile, almost all *EgrBZR* genes showed

differential transcript abundance levels in response to exogenously applied BR, MeJA, and SA, and salt and cold stresses. Besides, protein interaction analysis showed that all *EgrBZR* genes were associated with BR signaling directly or indirectly. These TFs were proposed as transcriptional activators or repressors of abiotic stress response and growth and development pathways of *E. grandis* by participating in BR signaling processes. These findings would be helpful in resolving the regulatory mechanism of *EgrBZR*s in stress resistance conditions but require further functional study of these potential TFs in *Eucalyptus*.

Keywords Brassinosteroids · BZR · *Eucalyptus* · Abiotic stress · Gene expression

Introduction

Brassinosteroids (BRs) refer to a class of plant steroid hormones, which are involved in various growth and developmental processes such as cell elongation, stress responses, photomorphogenesis, vascular development and senescence (Wei and Li 2016; Yang et al. 2011; Zhu et al. 2013). BRs signal through plasma membrane-localized receptor kinase Brassinosteroid-Insensitive 1 (BRI1) and other components of BR to modulate BRASSINAZOLE RESISTANT 1 (*BZR1*) and *BZR2* (also named BRI1-EMS-SUPPRESSOR1, *BES1*). *BZR1* and *BZR2* are then dephosphorylated (Tang et al. 2011) and localize to the nucleus to regulate transcription. Hence, *BZR1* and *BZR2* are considered to be the primary transcription factors in BR signaling (Wang et al. 2002; Yin et al. 2002; Zhao et al. 2002). *BZR1* and *BZR2* and other family members of BZR such as *BEH1-4* are exclusive to plants (Wang et al. 2002).

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The gain-of-function mutants of *bzr1-ID* in *Arabidopsis* show a reduced stature, with a slightly darker green and wider leaves, shorter petioles, shorter hypocotyls, delayed flowering and produce more leaves than wild-type (Wang et al. 2002). In *Arabidopsis*, genome-wide ChIP analysis demonstrated that *BZR1* and *BZR2* function either as activators or repressors, and regulate downstream targets by binding to BR response elements (BRREs) and E-boxes or additional promoter sequence elements and/or *BES1*- and *BZR1*-interacting proteins (Sun et al. 2010; Yu et al. 2011). Based on this analysis, it is established that *BZR1/BZR2* regulate numerous molecular links that are related to specific cellular and metabolic processes including growth responses, abiotic and biotic stress responses and light, jasmonic acid (JA) and abscisic acid (ABA) signaling pathways which evenly regulates BR biosynthesis (He et al. 2005; Sun et al. 2010; Yu et al. 2011). Recently, many studies have focused on the regulatory roles of *BZR1* and *BZR2* in BR signal transduction which interacted with other proteins to regulate transcription in *Arabidopsis*. *BZR1-PIF4* (phytochrome-interacting factor 4) interaction controls a core transcription network, which enables co-regulation of plant growth by steroid and environmental signals (Oh et al. 2012). Also, *BZR1-HY5* interaction regulates plant photomorphogenesis and cotyledon opening in *Arabidopsis* (Li and He 2016). *BZR1* could bind to the cis-elements of the FLOWERING LOCUS D (*FLD*) promoter to regulate flowering (Zhang et al. 2013b). *BZR1* along with *WRKY40* activate the expression of several other WRKY transcription factors that inhibit the immune responses (Rosa Lozano-Durán 2013). Few studies have demonstrated that *BZR2* in association with the transcription factors (*BIM1* or *HAT1*) regulate BR-induced genes or BR-repressed gene expression, in turn coordinating plant growth (Yin et al. 2005; Zhang et al. 2013a). *BZR2* directly binds to BRRE present in the *BRL3* promoter region, and modulates its transcription in different subsets of cells in the root apex (Salazar-Henao et al. 2016). Besides, *BZR1* and *BZR2* are responsible for inhibiting glucosinolate biosynthesis (Guo et al. 2013).

In contrast, little have been studied about *BEH1-4* that were thought to act redundantly with *BZR1* and *BZR2* (Jennifer Lachowiec 2016). Although *BZR* gene family is crucial in the embryonic stem development in *Arabidopsis*, only *BEH4* modulate the developmental robustness and chaperone *HSP90* which maintained robustness of embryonic stem length via *BEH4* (Jennifer Lachowiec 2016). The functions of BZR genes as well as their role in regulating genes in plants have been elucidated largely in *Arabidopsis* and rice. However, only fragmentary details about BZR characteristics and functions are known in other plant species such as *Petunia hybrida* (Verhoeef et al. 2013), *Glycine max* (Zhang et al. 2016), *Brassica rapa* (Saha et al.

2015) and *Zea mays* (Manoli et al. 2018). The characteristics and regulatory mechanisms of BZR genes in any woody plant species especially in the stress responsive environment still remain to be investigated.

Eucalyptus grandis are one of the most widely planted hardwood forest trees in the world due to their remarkable adaptability, fast growth, and superior wood properties (Myburg et al. 2014). However, *Eucalyptus* are susceptible to a range of biotic and abiotic stresses including salt and frost damage (Tibbits et al. 2006; Nasim et al. 2008), which can inhibit growth, reduce production and have significant effects on local economies. Thus, it's very important to understand the molecular mechanisms underlying the tolerance of *Eucalyptus* to biotic and abiotic stresses. Recent genome sequencing of *E. grandis* offered the possibility of genome-wide analysis of BZR gene family (Myburg et al. 2014). Based on the significance of BZR genes in plant growth and development and adaption to the environments and lack of any genome-wide systematic analysis of *E. grandis* BZR genes, the BZR gene family was selected for a systematic analysis in *E. grandis*.

In this study, we analyzed the genomic localizations, protein motif structures, phylogenetic relationships, and structures of candidate BZR genes in *E. grandis*. In addition, their tissue-specific expressions in different organs, genome-wide expression profiling of these genes in different tissues of *E. grandis* clone GL1 were also carried out. The expression patterns of these genes in response to salt, cold, BR, methyl jasmonate (MeJA) and SA treatments were further investigated. Finally, interaction network of homologs of six EgrBZR TFs and related genes in *Arabidopsis* was analyzed. Together, these findings suggest that EgrBZR genes play multiple roles during stress resistance in *E. grandis*.

Materials and methods

Identification and annotation of BZR gene family in *E. grandis*

A search was conducted on TAIR (<http://www.arabidopsis.org>) using the keyword “BZR”. We also searched from Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) by protein homologous alignment analysis using *Arabidopsis thaliana* BZR gene family members as query sequences. Protein sequences and coding sequences (CDS) of the identified *E. grandis* BZR TFs were obtained. To avoid the omission of BZR proteins in *E. grandis*, keywords were searched in NCBI (<http://www.ncbi.nlm.nih.gov/>), UniProt (www.uniprot.org) and PlantTFDB v3.0 (<http://planttfdb.cbi.pku.edu.cn/>). The sequences of all identified BZR proteins were subjected to SMART ([!\[\]\(6059a5aa8b4ca7bb793408023d6c6e42_img.jpg\) Springer](http://</p>
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smart.embl-heidelberg.de/) or Pfam (<http://pfam.sanger.ac.uk/>) analysis to confirm the presence of ‘DUF822’ domain (PF05687). The subcellular localizations of EgrBZR proteins were predicted using Plant-mPLoc (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>). The primary structures of the genes which included molecular weight and theoretical isoelectric point (pI) were analyzed using ProtParam (<http://web.expasy.org/protparam/>). The numbers of intron and exon were searched in Phytozome. Positional information about all *EgrBZR* genes along the 11 chromosomes of *E. grandis* was obtained from the EucGenIE (<http://www.eucgenie.org/>) (Myburg et al. 2014). The gene positions along 11 chromosomes were drawn by MapChart 2.30 (Voorrips 2002). To identify the putative cis-acting regulatory elements of *EgrBZR* family, 1500-bp upstream DNA sequences before the translation initiation codon (ATG) was analyzed using PlantCARE (Lescot et al. 2002).

Phylogenetic and conserved motif analysis of BZR genes in *E. grandis*

The BZR protein sequences were analyzed by MEME software Version 4.11.2 (<http://meme-suite.org/tools/meme>). A MEME search was executed with default parameters except that the maximum number of motifs was set to 10 and motif width was set from 6 to 200. Multiple protein sequences of the *BZR* genes in *E. grandis* were aligned using Cobalt (<https://www.ncbi.nlm.nih.gov/tools/cobalt>) with default parameters. To study the phylogenetic relationship of *Eucalyptus* BZR proteins along with their counterparts in *Arabidopsis* and *Populus*, full-length BZR protein sequences were obtained from TAIR (<http://www.arabidopsis.org>) and Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>), respectively. Multiple sequence alignment was performed, and phylogenetic tree was plotted using Cobalt by the Neighbor-joining method.

Plant material and treatments

E. grandis clone GL1 shoots were grown by tissue culture in pots in the greenhouse of Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou, Guangdong, China for 4 months. Four to eight fully expanded young leaves beneath the apex were taken for hormone and stress treatments, and the shoots were 25–35 cm long during that time. Hormone treatments were carried out by spraying leaves with 100 nM BR, 100 μ M salicylic acid (SA) and 100 μ M MeJA, and then the fully expanded young leaves were sampled at 0, 1, 6, 24 and 168 h post treatment. A salinity stress treatment was carried out by irrigating plants with 100 mM NaCl in the pots

followed by sampling leaves at 0, 1, 6, 24 and 168 h post treatment. A cold stress treatment was performed by placing plants into a 4 °C growth chamber and then the leaves were harvested at 0, 1, 6, 24 and 48 h post treatment. At the stipulated time point of each treatment, at least three leaves from three separate plants were combined to form one sample, and all of the treatment experiments were performed in triplicates. All these plant samples were immediately frozen in liquid nitrogen and stored at – 80 °C for total RNA extraction.

RNA isolation and Real-time quantitative PCR

Total RNA samples were extracted from leaves using the Qiagen Plant RNA Kit (USA). First-strand cDNA was synthesized by reverse transcription of 1.0 μ g total RNA using SuperScript III (Invitrogen) and cDNA products were diluted 25-fold for qRT-PCR. Gene-specific primers for each *EgrBRZ* gene were designed using Primer3 (Supplementary Table S1). Real-time quantitative PCR was conducted using SYBR green (TaKaRa Biotechnology) on LightCycle 96 real time-PCR machine (Roche) with a final volume of 20 μ l per reaction. Each reaction mixture contained 10.0 μ l SYBR Premix ExTaq II (TaKaRa Biotechnology), 2.0 μ l cDNA template, 0.8 μ l of each primer (1.0 μ M), and 6.4 μ l sterile distilled water. Each reaction was performed in triplicates. Cycling parameters were conducted at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. Melt-curve analyses were performed using a program at 95 °C for 15 s and then a constant increase from 60 °C to 95 °C. The *EgrEF2* gene was used as an internal reference gene (de Oliveira et al. 2011).

Results

Sequence analysis and phylogenetic classification of BZR in *E. grandis*

Six BZR TFs in *E. grandis* were identified, they were designated as *EgrBZR* TFs that included one *EgrBZR*, three *EgrBEH*, and two *EgrBAM* genes (Table 1). The predicted sizes of the 6 *EgrBZR*s ranged from 309 to 699 amino acids (34.13–77.95 kDa), and the predicted isoelectric points varied from 5.61 to 9.39 (Table 1). Analysis of the protein domain organization showed that all 6 *EgrBZR* TFs contained the ‘DUF822’ domain, which is a characteristic of BZR TFs. Among the 6 *EgrBZR* TFs, the coding sequences of 4 *EgrBZR* genes were disrupted by a single or double introns and exhibited similar structure and splicing patterns, while *EgrBAM7* and *EgrBAM8* contained 9 introns based on the *E. grandis* whole-genome sequence. The *EgrBZR* exon–intron patterns were similar to those of their

Table 1 Characteristics of BZR genes in *E. grandis*

Gene name	Locus	ORF (bp)	No. of introns	Protein				Sub-cellular localization
				Length (aa)	BZR domain position	PI	MW (kDa)	
EgrBZR1	Eucgr.F01541	930	1	309	11–158	9.39	34.13	Nucleus
EgrBEH1	Eucgr.H01239	963	1	320	5–143	9.39	34.7	Nucleus
EgrBEH3	Eucgr.D00428	963	2	320	3–141	9.04	34.32	Nucleus
EgrBEH4	Eucgr.F03236	963	2	320	3–135	8.76	34.53	Nucleus
EgrBAM7	Eucgr.E00139	2004	9	667	61–210	5.53	77.95	Nucleus
EgrBAM8	Eucgr.D02573	2100	9	699	75–226	5.61	75.36	Cytoplasm, Nucleus

homologs in *Arabidopsis*. Among the 6 *EgrBZR* genes, only *EgrBAM8* was predicted to have double splicing variants which were different from the proportion of alternative transcripts found in *Arabidopsis* and *Populus*.

Subcellular localization of the *EgrBZR* TFs was predicted by Plant-mPLOC. All *EgrBZR*s were identified to have nuclear localization (Table 1). One exception was in case of *EgrBAM8* which was localized in both cytoplasm and nucleus. Motif finding identified 10 motifs which were characteristics of *EgrBZR* TFs (Fig. 1). Motif finding and multiple sequence alignments revealed that all *EgrBZR* genes have BRASSINAZOLE RESISTANT1 (*BZR1*)-type DNA binding domains (DUF822) in their N terminal region which was represented by motif 1 (Figs. 1, 2). Most of the BZR TFs harbored the serine (S)-rich phosphorylation sites and a PEST domain that were represented by motifs 5 and 6, respectively. Motif 7 was a characteristic of the C-terminal domain. Multiple sequence alignments showed that this region was highly conserved among all the proteins.

To identify the putative cis-acting regulatory elements in *EgrBZR* TFs, about 1500-bp DNA sequences upstream of the start codon (ATG) were critically examined. The identified cis-elements included stress-responsive elements, as well as those involved in light response (e.g., ACE, G-box, Box I, Sp1, and Box 4), hormone response (e.g., TGA-element, TCA-element, ABRE, CGTCA motif, and TGACG-motif), stress response (e.g., HSE, LTR, Box-W1 element, and GC-motif), growth and development (e.g., circadian, Skn-1 motif, and GCN4 motif), and transcription (e.g., 5'UTR Py-rich stretch, and MBS element) (Supplemental Table S2).

The BZR family in *E. grandis* was contracted (contained only 6 members) when compared with 8 members in *Arabidopsis*, 12 members in *Populus*, and 15 members in *B. rapa* (Saha et al. 2015). To investigate the phylogenetic relationships of BZR family members, a phylogenetic tree was constructed by using deduced full-length amino acid sequences of BZR from *Eucalyptus*, *Arabidopsis* and *Populus* (Fig. 3). In the phylogenetic tree, the BZR members fell into 2 groups. BZR and BZR-homologs (BEH), which were identified as close homologs, were clustered into one group. It was interesting that only one *EgrBZR* was formed as a tight group with *BZR1* and *BZR2/BES1*, and it was designated as *EgrBZR1*. Another 3 BZR TFs were closely grouped with *Arabidopsis* BEH1, BEH3 and BEH4 and were designated as *EgrBEH1*, *EgrBEH3*, *EgrBEH4*, respectively. The other group included BAM7 and BAM8 which encoded a beta-amylase-like protein with a BZR domain and were designated as *EgrBAM7* and *EgrBAM8*.

In silico mapping of the gene loci showed that the 6 *EgrBZR* genes were evenly distributed on chromosomes 4, 5, 6 and 8 of *E. grandis*, with 1–2 genes per chromosome (Fig. 4). Similarly, in *Populus*, the *PtrBZR* genes were presented only on 9 of the 19 chromosomes, with 1–2 genes per chromosome, whereas in *B. rapa*, 15 BZR genes were scattered on 7 of the 10 chromosomes (Saha et al. 2015). Although 4 *EgrBZR*s (*EgrBZR1*, *EgrBEH1*, *EgrBEH3*, and *EgrBEH4*) shared greater than 50% similarity to each other, tandem and segmental duplications of *EgrBZR* genes were not detected based on the *Eucalyptus* genome data analysis (Myburg et al. 2014).

Fig. 1 Schematic representation of motifs distribution identified in *E. grandis* BZR proteins. Different motifs are indicated by different colors. The names of all members are shown on the left side of the figure, along with their phylogenetic relatedness

EgrBZR1	1	MTS[4]SSATRRRPSWRERENNRERRRRRAVAAKVYSGLRAQGNYNLPKHCDNNEVLKALCAEAGWVVEEDGTT	77
EgrBEH1	1	M— AGGASGRIPPTWKERENNRERRRRRAIAAKIYTLRAQGNYPKHCDNNEVLKALCTEAGWVVEEDGTT	71
EgrBEH3	1	MTS ———GSRPTWREERENNRERRRRRAVAAKIFSGLRMYGNYKLPKHCDNNEVLKALCDEAGWIVEADGTT	69
EgrBEH4	1	MTS ———GTRPTWKERENNRERRRRRAIAAKIFAGLRMYGNYKLPKHCDNNEVLKALCKEAGWVVEEDGTT	69
EgrBAM7	1	[74]— ———GARRSRPLEEKERTKLREHRRRAITARILAGLRHGNYNLRVRADINDVIAALAREAGWVLPDGT	141
EgrBAM8	1	[50]TAA[4]AVPGKGFEREKEKERTKLREHRRRAITSRLAGLRQYGNFPLPARADMNDVLAALAREAGWVEADGTT	127
DUF822 domain			
EgrBZR1	78	Y—RKGCRLLP—IDIPGSSYRATPYSSQ NPSPLSSSFSPSPSYQVSPSSSFSPSPTRINNAASNLPLYLRN	147
EgrBEH1	72	Y—RKGSKPP—GEIMGASANISACSSY QPSPQSSAFSPVPVSYHASPTSSSFSPSPTRFDANPSSYLLPFLRN	141
EgrBEH3	70	Y—RKGCKPVEHVDVARSETVSPSSY YPT—HNSPFGSSFPSPASSFYAAN—ANAD—GSFLIPWLKN	133
EgrBEH4	70	Y—RKGCRPLERMDIMGSTASPCSSY HPSPCASYNPSPASSFPSPARSYPGPHGNPD—ADSLIPWLKN	139
EgrBAM7	142	FpsrSQGSKP—ISGTSAAVTPPSSE[17]YGSPVDYNASRMKGVYVPNSSPYDSSSRGQSQISSALGDGGEPLN[11]	238
EgrBAM8	128	Y—RQSPPSQLATFSARSMDSPPASAAS[14]SAVMRMDLSLSPASLDVSVIAEKDPKSVKYGNASPINSV—[3]	209
Serine rich phosphorylation			
EgrBZR1	148	—A—IPSSLPLRLISNSAPVTPPLSSPTSRNPKVPVNEAIKQSM—AAFNYPIFAASAPASPTRNRLPPA	215
EgrBEH1	142	IAS—IPTNLPLRLISSAPVTPPLSSPTSRNSKRKPDWESLNSSIHkSSFRHPLFAASAPSSPTRRQNMTPP	213
EgrBEH3	134	FSSASSASSSKLPHLYIPGSSISAPVTPHLSPTARSPIKTGLDDQSTRFGW—GERYSFMPSTTPSPGHQ—	205
EgrBEH4	140	LSSGSSASSSKLPHHLFIPGSSISAPVTPPLSSPTSRTPRAKNHWDHVAASMTW—TGQHNSVFPSTTPSPGRQ—	212
EgrBAM7	239	VDKQVIVDVP SKLPERDFAATPYIPVYVMLPLGVINVKSELVDPDGLLKQLRI—LKSLNVDG	300
EgrBAM8	210	LDAEQLIHDVSSVDHQNVFTDTEFVPVYVKLD—GTINNYCQLVDQEVVRQELNH—IKSLNADG	270
EgrBZR1	216	TIPECD[11]WINFQR—fAPPQIPTSTYNLMKPTM—PQKSLED—AGKEQE	270
EgrBEH1	214	TIPECD[11]WVSFHQnvaVAAAAPPSPFTNLVKSSS—QQGPLKDQVEQNSSLAWVASSDRA	281
EgrBEH3	206	ILPDPE WISRIQ—IPQGGPVSTFTLVSSNPFQSKQEVLAAGGSRTWTPGQSGTCSPAIAAADHTGDIPMSEV	278
EgrBEH4	213	VLPDSG WLT—GGPSSPTFSLVARNPFGFKEEAFSGGSRMWTGPGQSGTCSPAIAAGADLTSDIPMSDS	279
EgrBAM7	301	VTVDC—WWGVVE—AHAPQEYNWNGYRRLFQMVHELKFKLQVIMSFHECGGNVGDVCIPLPHWVAEIGRSNP	370
EgrBAM8	271	VVVC—WWGIVE—GWNPRQYVWSGYRELFHIREFKLKLQVVLAFHEYGGNDSGDFSVSLPQWVLEIGKENE	340
EgrBZR1	271	RGSDFEF—MRGQVKPWEGERIHEV—GMDDLELTLGSMKNRS*	310
EgrBEH1	282	WGSEFEF—ENGRVKPWEGERIHEI—GVDDLELTLGSSKARA*	321
EgrBEH3	279	MSNEFAFGVSGKGLVKPWEGERIHDEPGSDLELTLGSGSKCR*	321
EgrBEH4	280	MATEFAFGSNATGLVKPWEGERIHED—ASDDLELTLGNARTR*	321
EgrBAM7	371	DIFFTDREGRNPECLSWGIDKERVLRGRTAVEVYFDYMRSFRA[286]	700
EgrBAM8	341	DIFFADREGRNPECLSWGIDKERVLRGRTAVEVYFDLMRSFRT[284]	668
C-terminal domain			

Fig. 2 Alignment of deduced amino acid sequences of EgrBZR proteins. Solid boxes represent DUF822 and C-terminal domains, respectively. Black lines indicate serine rich phosphorylation sites

among the six EgrBZR proteins. Numbers at the left and right indicate the positions of amino acid residues

Fig. 3 Phylogenetic tree showing the relatedness of the deduced full-length amino acid sequences of six *EgrBZR* proteins and all BZR family proteins of *Arabidopsis* (prefixed with ‘At’) and *Populus* (prefixed with ‘Ptr’)

Fig. 4 Chromosomal locations of *E. grandis* BZR genes along chromosomes. Chromosome numbers (e.g., Chr04 to Chr06, and Chr08) are indicated above the chromosomes. The plus (+) and minus (–) signs following gene names represent forward and reverse orientation of the respective genes. Gene positions and chromosome sizes can be estimated using the scale (in Megabases, Mb) to the left of the figure

Organ-specific expression analysis

Expressions of 6 *EgrBZR*s were analyzed based on previously published data set, in which transcript expression was analyzed in xylem, immature xylem, mature and young leaf, phloem and shoot tip tissues by de novo transcriptome assembly of *E. grandis* x *E. urophylla* and then re-calculated the transcript expression in *E. grandis* with the release of genome sequence (Mizrachi et al. 2010; Myburg et al. 2014). The results showed that 6 *EgrBZR* genes could be detected in all the tested tissues including xylem,

immature xylem, mature leaf, young leaf, phloem, and shoot tips, and most of them were preferentially expressed in certain tissues (Fig. 5). The relative transcript accumulation of all the *EgrBZR* genes was presented as a heatmap, and the hierarchical clustering allowed us to group all the expression patterns into two distinct clusters (Fig. 5). *EgrBEH1* and *EgrBAM8* were grouped into one cluster and highly expressed in non-vascular tissues like young leaf, shoot tips, and mature leaf; while *EgrBZR1*, *EgrBEH4*, *EgrBEH3*, and *EgrBAM7* were grouped into another cluster and preferentially expressed in vascular tissues like phloem, xylem, and immature xylem. Besides, *EgrBEH4* and *EgrBAM7* also highly expressed in shoot tips and young leaf. Interestingly, *EgrBEH3* was preferentially expressed in phloem as compared with immature xylem and xylem.

Fig. 5 Expression analysis of six BZR genes in different tissues of *E. grandis* x *E. urophylla*. Color bars at the top indicate the expression levels: red represents high expression and green represents low expression

Expression analysis of *EgrBZR*s in leaves in response to hormonal treatments and various abiotic stresses

BR, MeJA, and SA have been extensively studied for their roles in the regulation of various aspects of biotic and abiotic stresses. To gain insights into the stress responses of *EgrBZR* genes, the transcript levels of all *EgrBZR* genes were examined by qRT-PCR under BR, MeJA, and SA treatment. The results showed that their expression modes were complex. During BR treatment, *EgrBEH3*, *EgrBEH4*, *EgrBAM7*, and *EgrBAM8* were dramatically down-regulated at 1 h while *EgrBZR1* and *EgrBEH1* showed no obvious changes or a slight increase (Fig. 6a). Whereas all other *EgrBZR* genes were increased at the transcriptional levels with a peak at 24 h, except *EgrBEH4*, which was almost down-regulated at all the durations. It was interesting to note that all *EgrBZR* genes showed approximately equal expression levels at 168 h when compared to that at 0 h. The expression levels of *EgrBZR1* genes exhibited a continuously high-level of transcript abundance in response to the MeJA treatment with a 143-fold peak at 6 h, but only *EgrBEH1* was greatly transcribed after 6 h treatment (Fig. 6b). However, *EgrBEH4* showed a similar trend with obvious repression during different treatment durations. In addition, the expression of *EgrBEH3* remained at a low level without any significant change during the treatment time compared with the control. It is obvious that almost all the *EgrBZR* genes were down-regulated in response to SA treatment (Fig. 6c). Among them, *EgrBZR1* and *EgrBEH4* shared similar expression patterns, which showed strong down-regulation at all durations of SA treatment and exhibited more than 2 to tenfold decrease, while *EgrBAM7* was dramatically down-regulated at 1–24 h. However, the expression levels of the remaining *EgrBZR* genes exhibited slight repression or no significant changes.

To further assess the functions of *EgrBZR* genes which were involved in plant defense to abiotic stresses, we analyzed the expression of *EgrBZR* genes in response to salt and cold treatment. All the analyzed genes exhibited differential expression in response to the two abiotic stress treatments. During NaCl treatment, the expression levels of most *EgrBZR* genes exhibited similar expression pattern (Fig. 7a). It was observed that all *EgrBZR* genes were obviously decreased at 1 and 168 h at the transcriptional levels. On the contrary, the expression levels of *EgrBAM7* and *EgrBAM8* were greatly increased at 6 and 24 h. In contrast to NaCl treatment, *EgrBZR* genes showed differential expression pattern in response to cold stress. All *EgrBZR* genes were down-regulated at 6 and 24 h at the transcriptional levels, except *EgrBEH1*, which showed slight repression but with no significant changes at 6 h (Fig. 7b). Then the expression levels of *EgrBZR1*, *EgrBEH4*, and *EgrBAM8* were increased significantly at 48 h

of treatment while *EgrBEH3* and *EgrBAM7* remained at a significantly low level.

Interaction network analyses of homologs of *EgrBZR* proteins in *Arabidopsis*

We investigated the interactions of 6 *EgrBZR* TFs and their association with *Arabidopsis* proteins using STRING software (Fig. 8). *EgrBZR1* was highly homologous to BZR1 protein of *Arabidopsis*, which was involved in BR signaling network. BRASSINOSTEROID-INSENSITIVE 2 (*BIN2*), *BRI1*, BRI1 SUPPRESSOR 1 (*BSU1*), EARLY FLOWERING 6 (*ELF6*), GENERAL REGULATORY FACTOR 10 (*GRF10*), DWARF 4 (*DWF4*), BRI1-LIKE1 (*BRL1*), BRI1-LIKE2 (*BRL2*) and BRI1-LIKE3 (*BRL3*) appeared to participate in this process. Meanwhile, *EgrBEH1* was homologous to *BEH1* which was related to cell expansion in the growing tissues. POTASSIUM TRANSPORTER 2 (*KT2*) was associated with this process. *EgrBEH3* and *EgrBEH4* were responsive to stress and were strongly associated with HEAT SHOCK TRANSCRIPTION FACTOR B4 (*HSFB4*) and TOPLESS-RELATED 3 (*F2P16.14*, *TPR3*), RELATED TO AP2 10 (*RAP2.10*) and ERF DOMAIN PROTEIN 10 (*ERF10*), respectively. *EgrBAM7* and *EgrBAM8* were involved in carbohydrate metabolism and transport and were associated with FRUCTOSE-2,6-BISPHOSPHATASE (*F2KP*), ALPHA-GLUCAN PHOSPHORYLASE 2 (*PHS2*), SUGAR TRANSPORT PROTEIN 14 (*STP14*), ADPGLC-PPASE LARGE SUBUNIT (*APL2*), 4-ALPHA-GLUCANOTRANSFERASE (*DPE2*), ALPHA-AMYLASE-LIKE 3 (*AMY3*), SUCROSE SYNTHASE 6 (*SUS6*), DOF ZINC FINGER PROTEIN 2 (*DOF2*). Besides these, all the *EgrBZR* gene functions appeared by BR signaling network.

Discussion

The BZR TFs were crucial transcription factors in BR signal transduction pathway and were involved in the regulation of various processes in plants by directly regulating the expression of many BR-responsive genes. Although BR signaling pathway is well studied in *Arabidopsis*, the regulatory mechanisms in dicots remain poorly understood. However, few studies revealed the role of BZR family in various processes. For example, *PhBEH2* might function as an important hub in the cross-talk among diverse signaling pathways in *Petunia hybrida* (Verhoef et al. 2013). Similarly, in *G. max*, the expression accumulation of *GmBZL2* (homolog of *BZR1*) increased seed number per silique in *Arabidopsis* (Zhang et al. 2016). With the availability of *E. grandis* genome recently

Fig. 6 qRT-PCR expression analysis of *EgrBZR* genes under BR, MeJA, and SA treatments in leaves of *E. grandis*. The error bars represent standard error of the means of three independent replicates. The asterisk indicates a significant difference ($P < 0.05$) compared with control group according to Duncan's Test

(Myburg et al. 2014), complete and accurate annotation of genes/proteins is a key starting point for functional studies of transcriptional factors and gene families. Hence, a comprehensive genome-wide analysis of the phylogenetic relationships and expression profiles of BZR gene families in *E. grandis* was reported. This study aimed to lay a foundation to carry out functional studies on these potential BZR TFs, which ultimately provides a wide basis for the

exploitation of these candidate genes for further genetic transformation.

Organ-specific expression of 6 *EgrBZR* TFs in *E. grandis* showed their ubiquitous expression in most organs and 4 genes were highly expressed in the vascular tissues. The expression patterns of these TFs make them as good candidates for further investigations in wood formation-related functions. Similarly, the organ-specific expression

Fig. 7 qRT-PCR expression analysis of *EgrBZR* genes under cold and salt treatments in leaves of *E. grandis*. The error bars represent standard error of the means of three independent replicates. The asterisk indicates a significant difference ($P < 0.05$) compared with control group according to Duncan's Test

patterns were also observed in *ZmBZR* genes in maize (Manoli et al. 2018). The expressions of *BZR1* were increased at 1 week after BR treatment, which reduced the lignification and altered cell wall carbohydrate biosynthesis in the secondary xylem of *Liriodendron tulipifera* (Jin et al. 2014). It is interesting that *EgrBZR1* was significantly up-regulated in response to exogenous BR treatment.

Meanwhile, differential expression patterns of all the *EgrBZR* TFs against cold, salt, BR, MeJA and SA stresses were observed. Similar conclusions were also observed in *BZR* genes of other species. In maize, six *ZmBZR*s showed up-regulation against N starvation, hypoxia, and salt stress, however, their expression levels were down-regulated under heat stress (Manoli et al. 2018). In *B. rapa*, all *BrBZR*s showed several folds up-regulation against exogenous ABA treatment, but they showed differential expression against salt, drought, and low-temperature stresses (Saha et al. 2015). Notably, almost all 6 *EgrBZR* TFs were predicted to have both abiotic and biotic stress responsive cis-acting elements in their promoter sequences. Similar results were observed in *BZR* TFs of maize and *B. rapa* (Manoli et al. 2018; Saha et al. 2015). Besides,

differential transcript abundance levels were observed when these TFs responded to salt, low temperature, drought, and exogenous ABA treatment (Saha et al. 2015). More importantly, all the *EgrBZR* TFs showed differential expressions in response to exogenous BR treatment, which indicated that these *EgrBZR* TFs might play a role in response to BR signal transduction in *Eucalyptus*. The results of our study were similar to the previous reports regarding the roles of *BZR* TFs in BR signal transduction (He et al. 2005; Yin et al. 2005). Additionally, *EgrBZR1* was up-regulated and showed similar expression patterns in response to exogenous MeJA and BR treatments. In our protein interaction study, *EgrBZR1* showed strong association with DWF4, which functions in the response to JA in addition to BR signaling (Kim et al. 2013). Likewise, *EgrBEH4* was down-regulated and similar expression patterns were exhibited under MeJA and BR treatments. This protein interacted with TOPLESS-related 3 which was a transcriptional co-repressor and negative regulator of JA responses in addition to participating in BR signaling (Pauwels et al. 2010). These results indicated that the

Fig. 8 Interaction network of homologs of six EgrBZR TFs and the related genes in *Arabidopsis*. Stronger associations are represented by thicker lines

EgrBZR TFs might have multiple stress resistance-related functions in *Eucalyptus*.

As shown in Fig. 8, *BRI1* was strongly associated with *EgrBZR1*. *SUS6*, which is a member of the key enzymes involved in sucrose metabolism and is also involved in the cellulose and starch biosynthesis (Baroja-Fernández et al. 2012), and *AMY3*, which is localized in the chloroplast and regulates starch degradation as a member of glucan hydrolases that cleave α -1,4-glucosidic bonds in starch (Seung et al. 2013), were also associated with *BRI1*. Besides, *BZR1*-BAMs (*BAM7* and *BAM8*) were verified in controlling plant growth and development through cross-talk with BR signaling (Reinhold et al. 2011; Soyk et al. 2014). Our results suggest that all *EgrBZR* TFs appear to function in growth and development or stress resistance by participating in BR signaling processes.

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