scientific reports

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Genome-wide analysis of the BoBZR1 family genes and transcriptome analysis in *Brassica oleracea*

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The BRASSINAZOLE-RESISTANT 1 genes play a crucial role as key regulators in Brassinosteroid (BR) signaling, which affects various plant developmental and stress-responsive aspects. Understanding regulatory mechanisms via *BZR1* in modulating target genes has become a main point in research on plant BR signaling networks. Despite this, the *BZR1* functioning in *B. oleracea* is not elucidated. A complete genome-wide analysis identified 12 *BZR1* genes in *B. oleracea*, categorized into three groups based on their gene motif and structural features. These *BoBZR1s* were found on eight different chromosomes. Synteny analysis between *B. oleracea*, Arabidopsis, and potato provided perception into their evolutionary characteristics. Promoter regions of *BoBZR1* family genes in *B. oleracea* have shown specific cis-elements associated with hormones, stress, and plant development. The expression analysis toward cuticular wax biosynthesis revealed that BoBZR1-1, BoBZR1-6, BoBZR1-7, and BoBZR1-10 were upregulated in response to cuticular wax biosynthesis. Differential expressions of BoBZR1 genes were observed for all seven different tested tissues. The whole study involved systematic characterization of the BoBZR1 family, and expression patterns, in BR signaling and its extensive involvement in developmental processes in *B. oleracea*. Results establish a theoretical foundation for deeper investigation of *BoBZR1* structure and functions in *B. oleracea*, specifically toward regulating plant stress.

Keywords Brassinosteroids, BZR1, chromosomal mapping, Expression pattern, *Brassica oleracea*

BRs are the most extensively dispensed plant growth-regulating steroidal phytohormones and play a critical role in developmental activities apart from other regulator hormones like cytokinin and gibberellin[1–3]. Brassinosteroid functioning is specific toward plant germination, growth, development, and reproductivity[4]. Various studies indicated the fundamental role of BRs in response to sudden temperature fluctuations and other abiotic stresses like frost and salinity[5,6]. Brassinosteroids (BRs) engage with kinase receptors on the membrane, giving rise to form tierce complexes involving BRI1 (brassinosteroid insensitive)- RK1 (receptor kinase)-and LR (leucine-rich) repeat receptors, serve as the starting point for the initiation of BR signaling within the plant cellular environment[7,8]. Subsequent pathways of kinases are progressively activated to carry out their function, which leads to the dephosphorylation and expression of BZR1 transcription factors that ultimately modulate the expression of various genes via binding to brassinosteroid responsive elements (BRRE)[9–11].

BZR1 protein and BES1 protein, both belonging to the same family, are integral constituents of the brassinosteroid signaling in various pathways in plants. These proteins display a notable similarity in their

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sequences, sharing an overall 88% homology. Notably, the domains of both BZR1 and BES1 share 97% sequence consistency. This close sequence resemblance underscores their functional relatedness in mediating the plant's response to brassinosteroid signaling[10]. BZR1 is involved in the modulation of gene expression through BR signaling pathways[12]. The BZR1 protein contains a greatly conserved DNA-binding domain, the N-terminal contains that nuclear localization sequence[11]. The BZR1 proteins exhibit 22–24 amino acids sequence spanning glycosylation sites targeted by Glycogen Synthase Kinase 3. Additionally, certain members of the BZR family possess a PEST (Proline-Glutamic acid-serine-threonine) motif, serving as a regulatory element for modulating protein stability[11]. The control of downstream BR response genes is maintained via BR through the specific binding to BRRE[13,14]. The interaction between BZR1/BES1 and PIF4 involves their ability to associate with the auxin response element ARF6. This interaction facilitates the promotion of ARF6 binding to SAUR15 and SAUR18, regulating cell elongation[15]. Ultimately, BZR1 is significantly involved in adapting plants to abiotic stresses[16,17].

A highly susceptible to fungal infection mutant bes1-D provides compelling evidence of BES1's direct involvement in the defense against pathogens[18]. BES1 cooperated with RD26, leading toward the inhibition of RD26 expression, subsequently suppressing drought responses[19]. Alternatively, PIF4 activates BR synthesis, leading to elevated BES1 levels and promoting thermosmorphogenesis[20]. Moreover, BZR1 contributes to enhanced freezing tolerance in Arabidopsis through both CBF-dependent & independent mechanisms[21]. This intricate network of interactions highlights the regulatory mechanisms orchestrated by BZR1 in regulating plant responses to drought, freezing, and temperature fluctuations.

B. oleracea holds significant importance in the realm of nutrition and agricultural practices[22,23]. Rich in essential components, white cabbage contributes to attaining a harmonious vegetable crop and is recognized for its potential health benefits, including antioxidant properties[24–26]. To date, BZR1 gene analysis has been reported in *B. rapa*[27], *Pyrus bretschneideri*[28], *Nicotiana benthamiana*[29], potato[30], maize[31] and Arabidopsis[32]. Yet, our knowledge regarding the evolutionary history and expression profiles of Brassinazole-resistant proteins (BZR1) in *B. oleracea* is currently constrained. Exploration through genome-wide analysis is indispensable for understanding the growth and development of *B. oleracea*. Hence, this investigation undertook to identify and characterize the BZR1 family in *B. oleracea*. The findings of this research establish a foundation for subsequent functional investigations of BZR1 genes in *B. oleracea*.

Materials and methods

BZR1 genes identification in *B. oleracea*

The amino acid sequence corresponding to the *BZR1* was identified from the tomato peptide genome using the identifier PF05687, obtained from the Pfam database (http://pfam.xfam.org/)[33]. The Phytozome platform (https://phytozome-next.jgi.doe.gov) was utilized to locate *BZR1* genes within the genomes of *B. oleracea*, employing this peptide sequence. Redundant genes were then filtered out to ensure non-redundancy. Subsequently, the non-redundant protein sequence was validated using the Motif-Finder online database and NCBI-CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)[34]. To eliminate redundancy, we removed duplicate genes. Subsequently, the selected genes underwent additional validation to ensure the presence of the BES_N domain utilizing default parameters on both the Motif-Finder online database and NCBI-CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)[35].

Determining physio-chemical properties and sub-cellular localization

Data regarding transcript IDs, gene localization, and gene and protein sequences were sourced from Phytozome. The Prot-Param online tool (http://web.expasy.org/protparam/) is employed to retrieve physio-chemical characters of *BoBZR1* family genes, encompassing molecular weight, protein length, isoelectric point[36], and instability index[37]. The forecasted subcellular localizations of *BoBZR1* genes were determined utilizing WoLF PSORT (https://wolfpsort.hgc.jp/). The heat map, created through TBTools, allows for visual inspection of localization features across different cell organelles, using output data from cello-life (https://cello.life.nctu.edu.tw/) for enhanced representation[38].

Evolutionary analysis and classification of *BZR1*

To observe the evolutionary dynamics of *BZR1* proteins in Arabidopsis, tomato, cucumber, potato and cabbage, the full amino acid sequences of these genes were aligned using MUSCLE, employing default settings[39]. A neighbor-joining tree was constructed using MEGA11, incorporating statistically reliable bootstrap replication no. of 1000[40]. The resulting phylogenetic tree facilitated the classification of *BZR1* transcription factors into various groups based on the tree's topology. For an enhanced visual representation, the aligned dataset was taken to iTol (https://itol.embl.de/personal_page.cgi) for the creation of a visually enriched and informative display[41].

Exon–intron distribution of *BoBZR1* and chromosomal positioning

To delineate the intron/exon architecture of the *BoBZR1* gene, genomic and CDS files were utilized that were sourced through Phytozome. Utilizing these sequences, an online server (http://gsds.cbi.pku.edu.cn/) was employed to visually represent and analyze the gene structure of the *BoBZR1* family genes[42].

Chromosomal localization of all genes denoted by their chromosome number and precise start & end position was obtained using Phytozome and ProtParam. All chromosome length was evaluated from the genome file using TBtools. Additionally, a comprehensive chromosomal map illustrating the positioning of the *BoBZR1* genes was generated using an online website (http://mg2c.iask.in/mg2c_v2.0/), following default settings for optimal visualization and analysis.

Comparative domain and conserved motif analysis

Comparative domain within the *BoBZR1* family was analyzed using the NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), employing default settings. Motif analysis of proteins within the *BoBZR1* was carried out utilizing the MEME suite (https://meme-suite.org/meme/)[43], and all other settings were kept at their default encompassing a lowest motif width of six and a highest width of fifty[44]. The TBTool software was utilized to visualize conserved domains and motifs, incorporating the hit-data, phylogenic data, and MEME-suite data[45].

Assessment of Cis-regulatory elements (CREs) in *BoBZR1* family gene promoters

Promoter regions were retrieved from phytozome to extract cis-regulatory elements. These elements have regulatory control over the functionality of target genes[46]. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was utilized to take CREs, spanning 5–20 base pairs within the promoter region[47]. Subsequently, the obtained data of measured specific regulatory elements were mapped in TBtools and Venn diagram was created using an online website Venny2.0.2 (https://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html).

Gene-duplication study and synteny analysis

BoBZR1 genes divergence time was assessed through Ka/Ks values. The software TBtools was utilized to ascertain the quantity of Ka/Ks substitution ratios. Paralogous gene pairs were subjected to Ka/Ks ratio calculations, providing insights into each gene pair's pace of evolutionary progression. To estimate the time of divergence (T), the Ks value was input into the equation $T = Ks / 2\lambda$, where λ equaled 9×10^{-9}[48]. For the synteny analysis, TBtools was utilized with default parameters, showing a comprehensive exploration of various gene duplication events.

Enrichment analysis and gene ontology

Gene Ontology analysis was conducted to validate the functions of BoBZR1 genes for additional confirmation of their functional roles[49]. Arabidopsis online database (https://www.arabidopsis.org/) was employed to elucidate the molecular functioning and their participation in diverse biological processes of genes. Additionally, a web tool (http://bioinformatics.sdstate.edu/go/) was employed to comprehend the roles played by BZR1 genes in *B. oleracea*. A p-value of 0.01 was employed to determine the significance of gene enrichment in the analysis[50].

BoBZR1 genes expression analysis

Transcriptome analysis of *BoBZR1* genes was undertaken to assess their involvement in cuticular wax development and expression patterns across various tissues examined to verify their functional relevance in response to both stress and growth stimuli.

Expression of BoBZR1 genes in glossy mutant Nwgl and wild-type

A cabbage (*Brassica oleracea L. var. capitata*) plant with a non-wax glossy (nwgl) phenotype was obtained from the inbred line G287. To produce F_2 mapping populations, this plant was crossed with the inbred lines G306 and G274. The cultivation occurred under natural field circumstances at the Shanghai Academy of Agricultural Sciences in Shanghai, China, throughout the regular growing season. High-throughput RNA sequencing data was utilized to assess the expression profiles in leaf samples obtained from a glossy mutant and its corresponding wild-type in cabbage. The experiment employed three replications for data collection. Subsequent analysis facilitated the identification of differentially expressed genes (DEGs) in nwgl leaves in comparison to the WT[51].

Tissue-specific expression of *BoBZR1* genes

Genes that were differently expressed that could be related to the developmental processes of plants were found using RNA-Seq expression data from seven different *B. oleracea* tissues[52]. To examine variations in BoBZR1s, expression was measured in numbers of fragments per kilobase of transcript per million mapped reads (FPKM) across seven distinct tissues.

Protein–protein (PPI) interaction

Utilizing STRING (https://string-db.org/), protein interaction (PPI) was analyzed through setting a 0.7 stringent confidence score[53]. The PPI network was established by integrating active interactions with a score of 0.4 from various sources. This interactome map facilitated the identification of key candidate genes participating in both physical and functional interactions[54].

Results

Identification of *BoBZR1* family gene members

Through this analysis, twelve *BoBZR1* genes in *B. oleracrea* were successfully identified. Subsequent to the exclusion of redundant sequences, the dataset was refined by eliminating repetitive elements. The 12 *BZR1* genes in *B. oleracea* were denoted as *BoBZR1-1* to *BoBZR1-12*, with each designation corresponding to its specific chromosomal location. The comprehensive analysis of their physiochemical characteristics encompassed an in-depth analysis. Parameters scrutinized included transcript ID, isoelectric point, molecular weight, polypeptide length (Table 1), and the forecasted sub-cellular location of these *B. oleracea* proteins (Fig. 1). The *B. oleracrea* protein length exhibited variation, spanning from 94 amino-acid (AA) in *BoBZR1-4* to 700 amino-acid (AA) in *BoBZR1-3*. Correspondingly, the molecular weights (MW) associated with these proteins ranged from 10.880 kDa to 79.09 kDa. The isoelectric point (pI) values showed diversity, ranging from 5.88 in *BoBZR1-1* to 11.11 in *BoBZR1-10*.

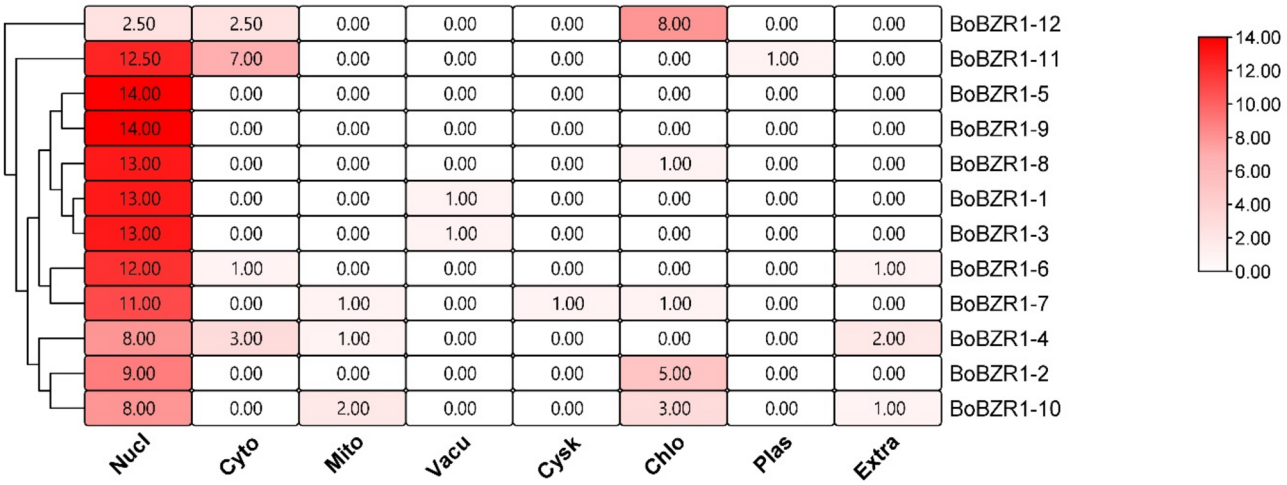

Fig. 1. Heat Map showing all 12 *BoBZR1* genes Sub-cellular localization. White-red represents the lowest to highest value of the gene in that specific organelle.

Transcript ID	Gene name	chr#	Location		Strand	AA	Molecular weight (kDa)	pl	Introns	Exons	Cellular localization
			Start	End							
Bol030219	BoBZR1-1	4	3,108,784	3,112,093	R	678	75.87934	5.88	9	10	Nucleus/cytoplasm
Bol026849	BoBZR1-2	5	14,902,839	14,903,937	F	330	36.09818	9.51	1	2	Nucleus
Bol022860	BoBZR1-3	2	30,645,425	30,648,487	F	700	79.09788	5.99	8	9	Cytoplasm
Bol024404	BoBZR1-4	7	41,219,910	41,220,195	F	94	10.88032	10.1	0	1	Nucleus
Bol018647	BoBZR1-5	7	47,454,546	47,455,848	R	305	33.16615	9.24	1	2	Nucleus
Bol016953	BoBZR1-6	7	37,749,528	37,750,758	R	285	30.68924	9.41	1	2	Nucleus
Bol027756	BoBZR1-7	6	2,451,478	2,452,496	R	302	33.1791	9.46	1	2	Nucleus
Bol039912	BoBZR1-8	6	32,437,750	32,439,307	R	333	36.18426	8.92	1	2	Nucleus
Bol028970	BoBZR1-9	1	1,153,098	1,154,534	F	303	33.01577	8.63	1	2	Nucleus
Bol009369	BoBZR1-10	1	6,830,075	6,830,463	F	106	12.5295	11.11	1	2	Nucleus
Bol044834	BoBZR1-11	8	36,226,172	36,227,601	R	322	35.3012	9.51	1	2	Nucleus
Bol013150	BoBZR1-12	8	21,265,789	21,266,945	F	337	36.63471	9.28	1	2	Nucleus

Table 1. A comprehensive examination of various attributes of BZR1 genes discovered in *B.oleracea*.

Phylogenetic relationship, structure and motif analysis of *BoBZR1* genes

A phylogenetic tree was constructed to clarify the phylogenetic relationships among *BZR1* proteins and explore the evolutionary dynamics of genes (Fig. 2a). This tree encompasses *BZR1* proteins from *B. oleracea*, as well as from Arabidopsis (16 proteins), tomato (9 proteins), cucumber (6 proteins), and potato (11 proteins). Utilizing the Arabidopsis *BZR1* families as a reference, we classified *BoBZR1* into 3 distinct classes: A, B, and C. Class-B turned out to be the biggest, having a total of 40 members. Proteins within the same clade generally share structural and functional similarities. Accordingly, the identified *BZR1* proteins are expected to exhibit comparable structures and functions, indicating functional coherence among clade members.

The structural organization of *BoBZR1* genes was examined by analyzing the intron–exon structures of 12 *BoBZR1* gene members (Fig. 2b). Exons and introns function as pivotal structural components that influence the intricate tapestry of evolutionary interrelations among genes[55]. The *BoBZR1* genes exhibited inconsistency in the number of introns and exons, ranging from one to ten. *BoBZR1-1* contained 10 exons (52.63%), *BoBZR1-3* had 9 exons (52.94%), while *BoBZR1-4* was entirely composed of exons (100%). Additionally, nine genes contained 2 exons (66.66%).

Domain structure was scrutinized inherent in their protein sequences (Fig. 2c). *BoBZR1* proteins analysis involved a detailed analysis of 15 identified motifs through the MEME program. Analyzing the *BZR1* protein family showed that the same group of proteins share the same motifs. Some genes contained fewer motifs, i.e. *BoBZR1-10*, *BoBZR1-4*, *BoBZR1-1* and *BoBZR1-3* contained 2, 2, 3 and 3 motifs, respectively. All the genes contained Motif 1 and Motif 3. The existence of similar motifs among various members of the *BZR1* proteins points towards the impact of gene expansion as a driving force in the evolutionary dynamics of these genes[56].

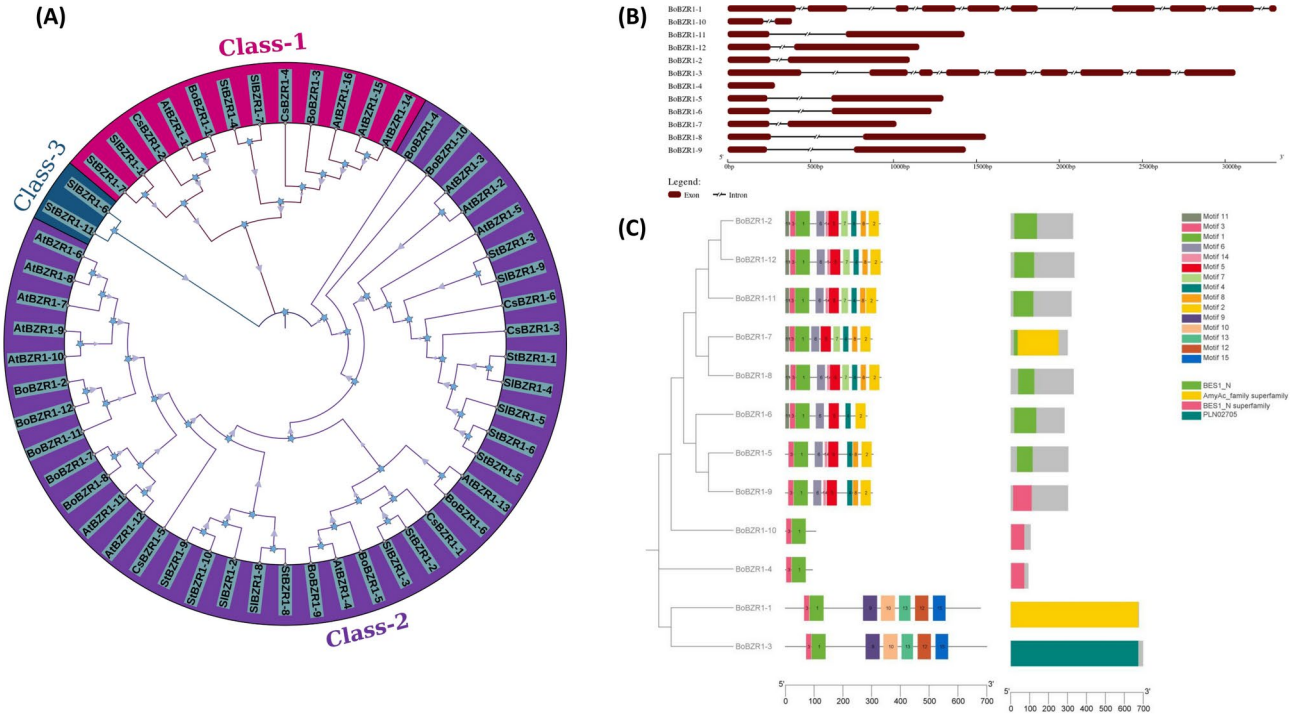

Fig. 2. (**A**) Evolutionary relationship in *BZR1* genes of *Solenum tuberosum*, *(A) thaliana*, *S. lycopersicum*, *C. sativus*, and *(B) oleracea*. (**B**) Intron-Exon structures of the *BoBZR1* genes. Red boxes show exons, while black lines show introns. (**C**) Motif distribution on *BZR1* proteins in *B. oleracea* with a phylogenetic tree. Each bar in the graph is assigned a distinct color code to differentiate between the various types of motifs.

Analysis of promoter Cis-element of *BoBZR1*

Cis-acting elements are non-coding DNA sequences in gene promoters that regulate gene transcription[57]. Twelve specific CREs within this region were identified that are highly involved in plant growth and developmental activities, stress and hormone responsiveness (Fig. 3). These include core elements, MBS (linked to drought inducibility), TC-rich repeats (involved in defense and stress responses), and ARE (responsible for anaerobic induction). Regarding hormonal regulation, there are elements such as TGACG-motif and CGTCA-motif (associated with methyl-jasmonate), ABRE (related to abscisic acid) and TGA-element (responsive to auxin). Additionally, gibberellin-related motifs (P-box) are identified, along with regulatory motifs for tissue-specific expression (e.g., GCN4_motif and CAT-box) and those associated with promoter and enhancer regions (e.g., CAAT-box). Notably, the element in promoter and enhancer regions emerges as the most abundant cis-acting element in the *BoBZR1* promoter region.

Chromosomal distribution and duplication analysis

The genomic positioning analysis of *BoBZR1* genes in *B. oleracea* revealed a markedly uneven distribution across eight chromosomes (Fig. 4). Chromosome 07 showed a notable concentration with three *BoBZR1* genes, while chromosomes 1, 6, and 8 each contained two genes. The remaining chromosomes predominantly harbored a single gene.

Synteny analysis of *BoBZR1* genes revealed a non-uniform distribution of most paralogous gene pairs across the *B. oleracea* genome, suggesting their emergence from whole-genome duplication or segmental duplication (Fig. 5a). However, three paralogous gene pairs situated closely on the same chromosome likely resulted from tandem duplication. Specifically, *BoBZR1-5*, *BoBZR1-6*, *BoBZR1-11*, and *BoBZR1-12* genes were identified as products of tandem duplication, while others were inferred to have arisen from whole-genome duplication or segmental duplication events. Multiple synteny analyses were conducted comparing the whole genomes of *B. oleracea*, *Solanum tuberosum*, and Arabidopsis (Fig. 5b). The duplicated genes between the chromosomes of these crops were highlighted by connecting threads. Seven *BoBZR1* genes were duplicated between *B. oleracea* and Arabidopsis, while six were duplicated between *B. oleracea* and *S. tuberosum*.

We utilized Ks (synonymous substitution rate) as a metric to track the dates of duplication blocks, employing it as a means to estimate the timing of duplication events. This approach enabled us to trace the origin of segmental duplications in *BoBZR1* genes (Fig. 5c), spanning from *BoBZR1-2_BoBZR1-12* around 18.47 million years ago (Ks = 0.332) to *BoBZR1-6_BoBZR1-12* approximately 259.61 million years ago (Ks = 4.673). For all four of the paralogous pairings in *B. oleracea*, the Ka/Ks ratio was found to be less than 1.

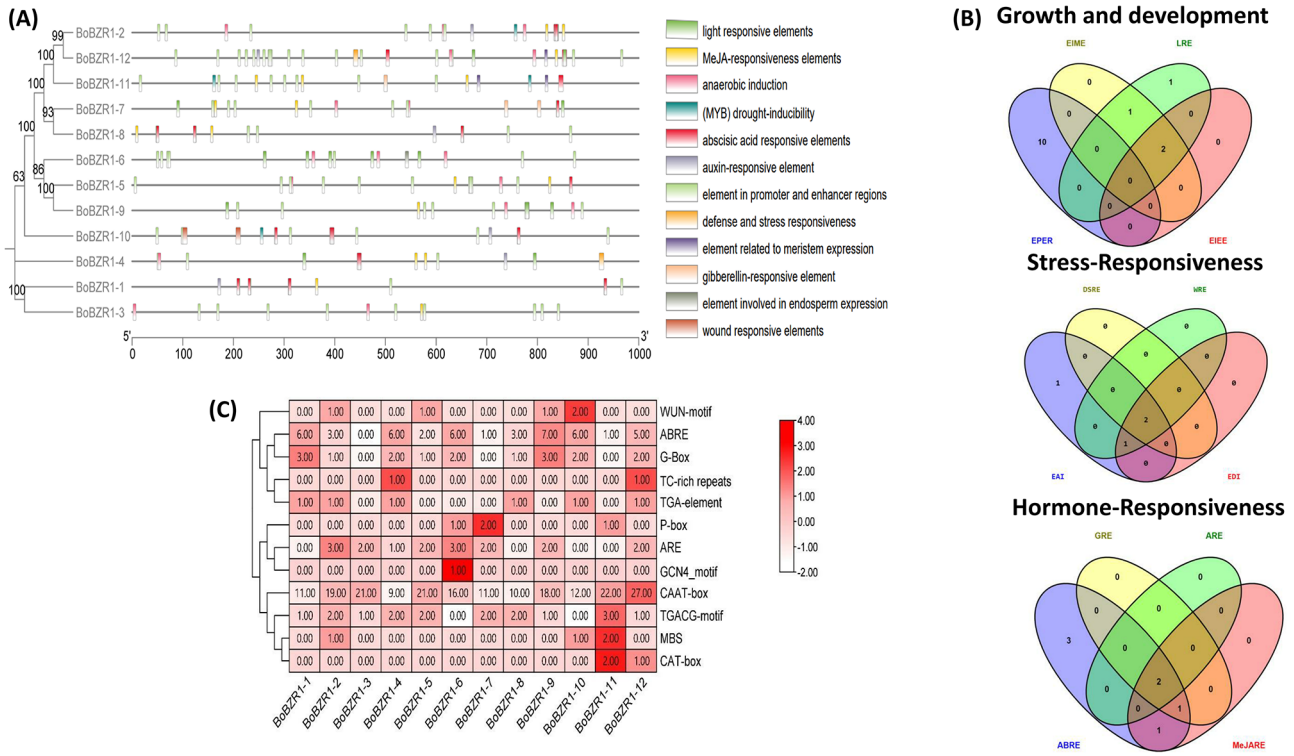

Fig. 3. (**a**) 12 *Cis*-regulatory elements in putative *BoBZR1* promoters which are linked to different plant developmental processes (**b**) Venn diagram depicting the ratio of growth and development, stress-responsive and hormone-responsive *Cis*-regulatory elements. Plant development responses involve elements associated with light responsiveness, elements in promoter and enhancer regions, endosperm expression and meristem expression. Hormone response elements exhibit reactions to abscisic acid-responsive elements (ABA), gibberellin-responsive element, auxin-responsive elements and MeJA-responsiveness elements, while stress responses include elements related to anaerobic induction, defense and stress-responsive elements, wound responsive elements (MYB) and drought-inducibility. (**c**) Heatmap of 12 specific cis-regulatory elements related to growth and development, harmones and stress.

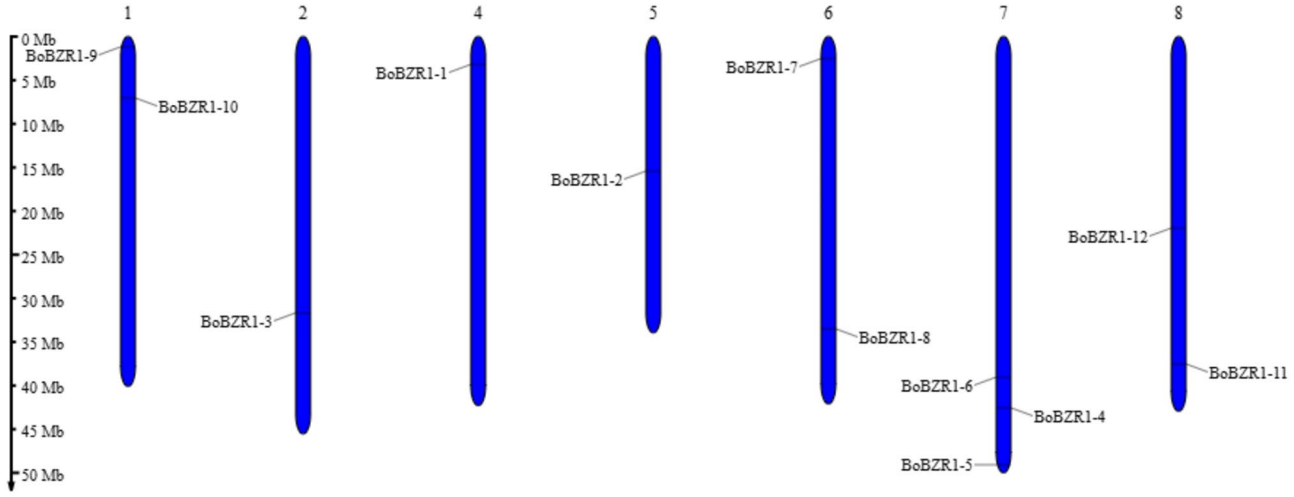

Fig. 4. Distribution of *BoBZR1* genes on *B.oleracea* chromosomes. The scale on left side in Mb indicates various chromosome lengths.

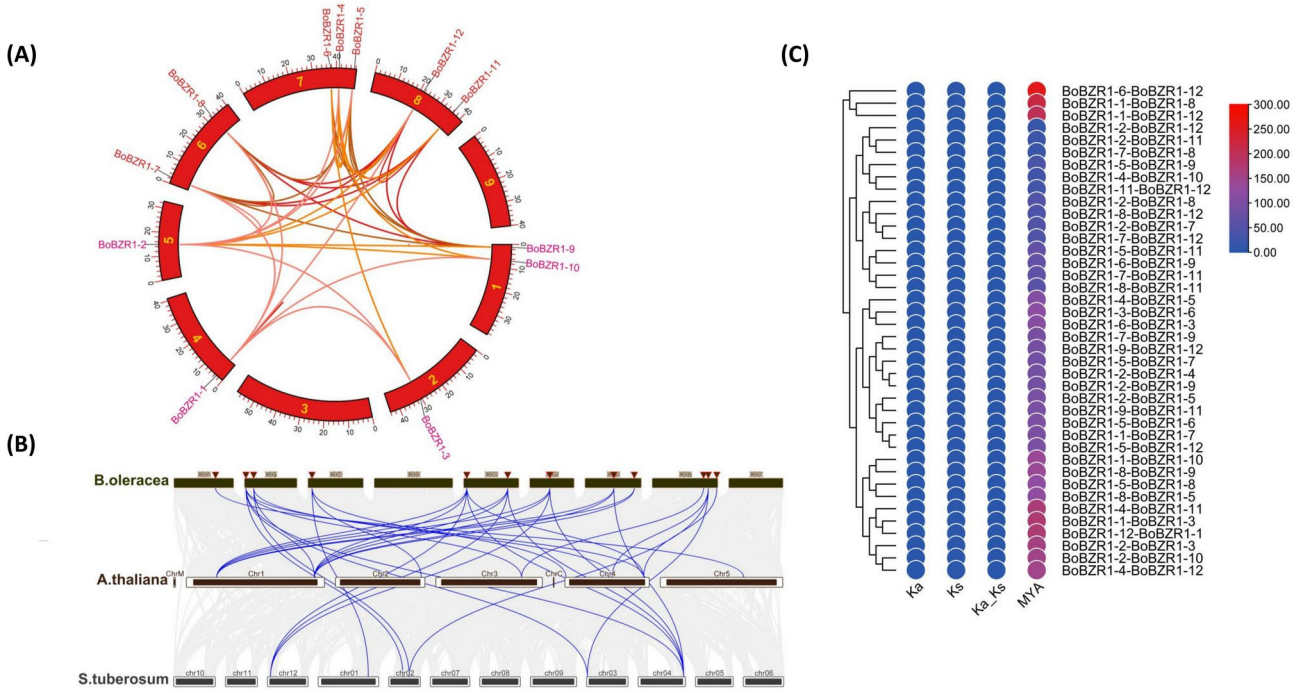

Fig. 5. (**A**) Genome-wide synteny analysis of *BoBZR1* genes showing the dominance of segmental duplication and rare occurrence of tandem duplication. (**B**) Multiple synteny analysis of *B.oleracea*, Arabidopsis and Potato. Blue lines showing the duplicated genes in the respective genomes. (**C**) The time of gene duplication for different paralogous pairs of BoBZR1 genes was estimated using TBtool software based on Ka (nonsynonymous substitutions) and Ks (synonymous substitutions) values.

Gene ID	Arabidopsis orthologue	Biological process	Location	Molecular function
BoBZR1-1	AtBZR1-1 (AT2G45880.1)	Regulation of shoot system development	Extracellular region, nucleus	DNA-binding transcription factor activity, amylopectin maltohydrolase activity, protein binding
BoBZR1-2	AtBZR1-6 (AT1G19350.3)	DNA-templated transcription, brassinosteroid-mediated signaling pathway	Chloroplast, cytoplasm, cytosol, nucleus	Cis-regulatory region sequence-specific DNA binding, protein binding, transcription regulator activity
BoBZR1-3	AtBZR1-14 (AT5G45300.3)	Regulation of shoot system development	Extracellular region, nucleus	DNA-binding transcription factor activity, protein binding
BoBZR1-4	AtBZR1-2 (AT4G18890.1)	Regulation of DNA-templated transcription	Nucleus	DNA-binding transcription factor activity, protein binding
BoBZR1-5	AtBZR1-4 (AT4G36780.1)	Regulation of DNA-templated transcription	Nucleus	Protein binding
BoBZR1-6	AtBZR1-13 (AT3G50750.1)	Regulation of DNA-templated transcription	Nucleus, plant-type vacuole	DNA-binding transcription factor activity, protein binding
BoBZR1-7	AtBZR1-12 (AT1G75080.2)	DNA-templated transcription	Cytosol, nucleus	Cis-regulatory region sequence-specific DNA binding, protein binding
BoBZR1-8	AtBZR1-11 (AT1G75080.1)	Brassinosteroid-mediated signaling pathway	Cytosol, nucleus	Plant ovule development, regulation of DNA-templated transcription
BoBZR1-9	AtBZR1-4 (AT4G36780.1)	Regulation of DNA-templated transcription	Nucleus	Protein binding
BoBZR1-10	AtBZR1-2 (AT4G18890.1)	Regulation of DNA-templated transcription	Nucleus	DNA-binding transcription factor activity, protein binding
BoBZR1-11	AtBZR1-6 (AT1G19350.3)	UV protection, brassinosteroid-mediated signaling pathway	Chloroplast, cytoplasm, cytosol, nucleus	Cis-regulatory region sequence-specific DNA binding, protein binding, transcription regulator activity
BoBZR1-12	AtBZR1-10 (AT1G19350.6)	UV protection, brassinosteroid-mediated signaling pathway	Chloroplast, cytoplasm, cytosol, nucleus	Cis-regulatory region sequence-specific DNA binding, protein binding, transcription regulator activity

Table 2. Arabidopsis orthologue of *B.oleracea* and their expression pattern analysis in BoBZR1 gene family.

Orthologue identification and enrichment analysis

The functions and subcellular localization of *BoBZR1* genes orthologous with Arabidopsis were determined at the molecular level. Simultaneously, broader biological processes involving these genes were identified through available datasets (Table 2). A fold enrichment graph was generated using ShinyGO v0.741 to visualize the overlapping effects of these genes on various biological processes (Fig. 6). The chart was employed to acknowledge genes' involvement in different biological functions in the *B. oleracea*. In the biological process category, most of the *BZR1* proteins were found to be involved in regulating DNA-templated transcription. In

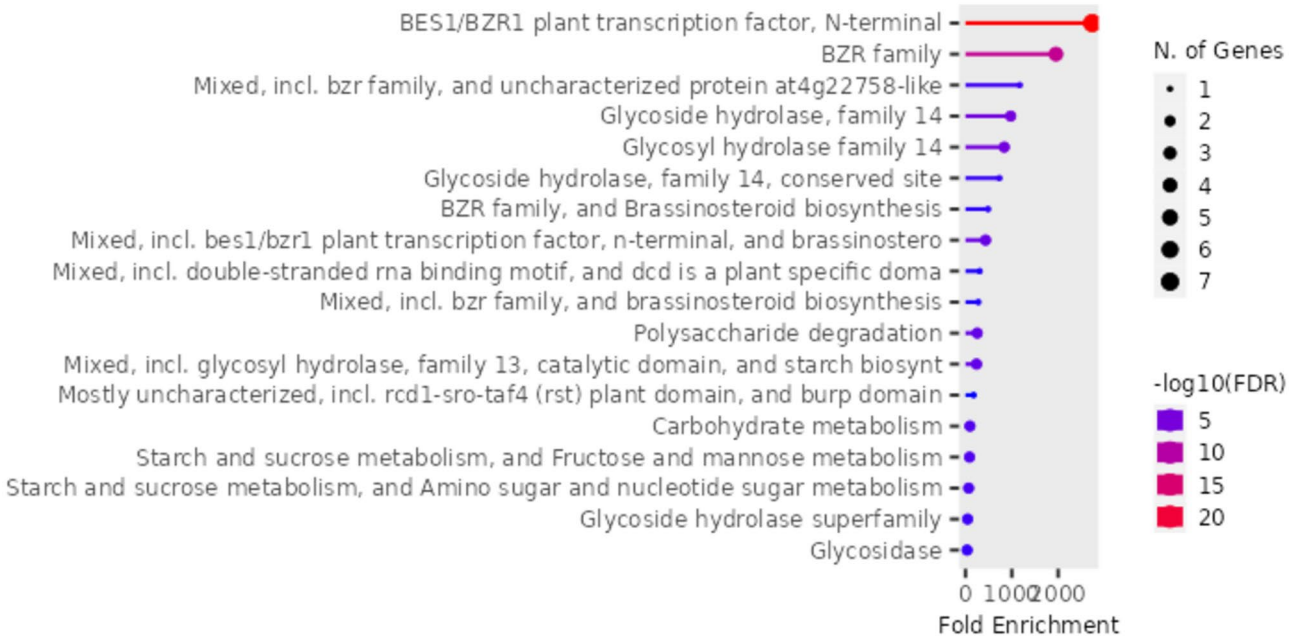

Fig. 6. The Fold Enrichment chart illustrates the shared functions of *BoBZR1* genes, with red-colored dots indicating a higher number of genes involved in a specific process, while smaller blue dots represent fewer genes associated with that process. The chart provides a visual representation of the enrichment of functions among the overlapping *BoBZR1* genes.

terms of molecular functions, these genes predominantly participate in sequence-specific DNA binding, protein binding, and transcription regulator activity.

Transcriptomic analysis

BoBZR1 genes expression towards cuticular wax biosynthesis

In this experiment, two varieties of *B. oleracea*, namely the wild-type and glossy mutant (nwgl), were cultivated. Comparative analysis was done between both varieties, involving the calculation of p-values and log2fold change. *BoBZR1-7* depicted significant differential expression. Genes like *BoBZR1-1*, *BoBZR1-6*, *BoBZR1-7* and *BoBZR1-10* showed considerable upregulation while the other genes were downregulated from wild-type and glossy mutant (Fig. 7a).

Tissue-specific *BoBZR1* genes expression and correlation analysis

A heatmap of RNA-Seq gene expression profile in different tissues obtained for all 12 *B. oleracea BZR1* genes was constructed (Fig. 7b). The tissues included in the analysis are callus, root, stem, leaf, flower, and silique. In all seven tested tissues, the *BoBZR1* member's expression was consistently observed. All the genes depicted significant expression across various tissues. The results depicted that the genes *BoBZR1-1*, *BoBZR1-3*, *BoBZR1-4*, *BoBZR1-7*, *BoBZR1-8*, *BoBZR1-10* and *BoBZR1-11* were highly expressed in stem while lowest expression in flowers. Moreover, *BoBZR1-2* exhibited maximum expression levels in the leaf compared to other tissues. Conversely, *BoBZR1-6* demonstrated lower expression in the root.

Protein–protein interaction

The interaction among proteins plays a crucial role in facilitating various essential plant functions and processes, including signal transduction pathways, plant development, and physiological and pathological processes[58]. In the case of *BoBZR1* proteins, their protein-protein interactions were investigated using the STRING database. The analysis revealed interactions among *BZR1* proteins themselves as well as with various other proteins in *B. oleracea*, indicating their involvement in diverse cellular processes and pathways within the plant.

PPI network exhibits an average local clustering coefficient of 0.269 (Fig. 8). This value depicts a medium clustering level, indicating that proteins in the network tend to interact with other proteins that are also interconnected. Seven genes were interrelated forming one cluster while *BoBZR1-1* and *BoBZR1-3* were interrelated to each other forming a separate cluster. The genes like *BoBZR1-4* and *BoBZR1-10* show no relation with other genes.

Discussion

BR, a plant-specific steroidal hormone, exhibits a vast range of stress-resistance functioning that influences different growth processes[59]. Additionally, the BR signal transduction system is essential for plant development. *BZR1* proteins maintain interlinks in BR and other different pathways of signaling[60]. *PhBEH2* is suggested to serve as a key component facilitating crosstalk between signaling pathways in the Petunia hybrid[61]. The *BZR1*

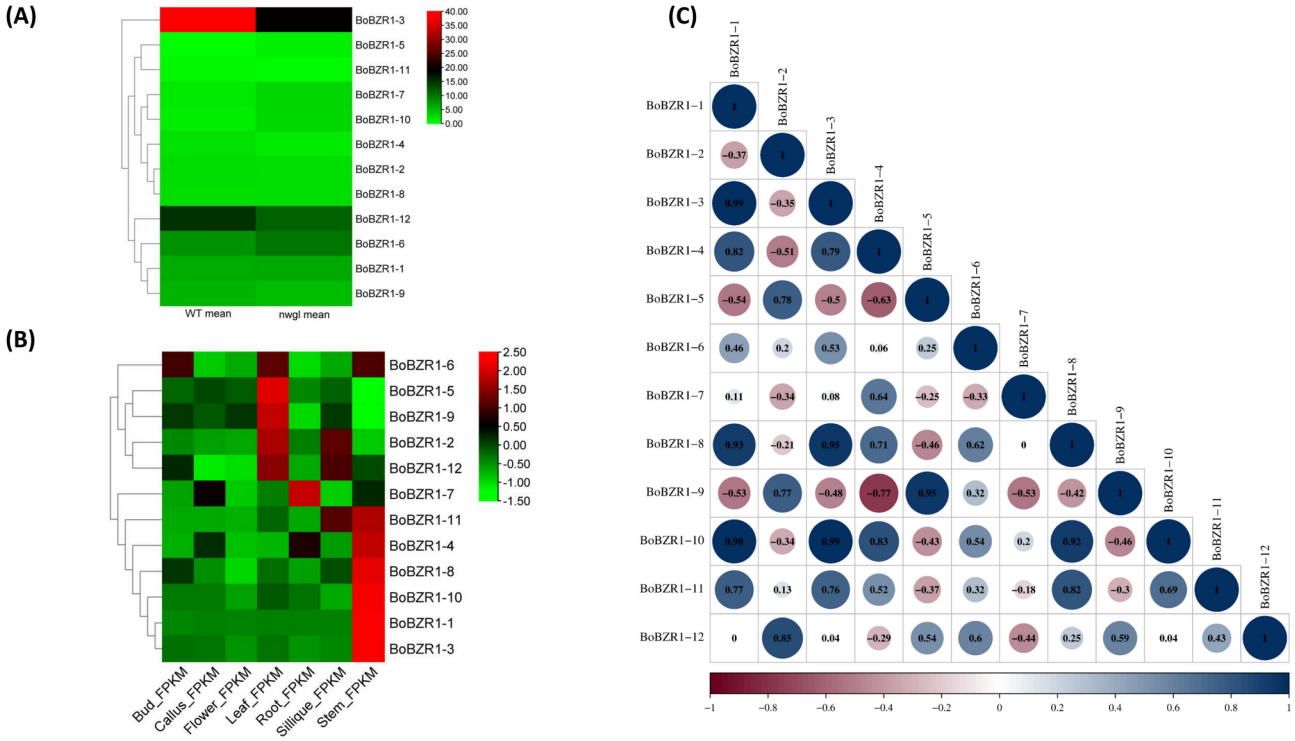

Fig. 7. (**A**) Relative expression profile of *BoBZR1* genes from wild type to mutant type *B.oleracea* varieties. (**B**) Relative expression profile in FPKM of the *BoBZR1* genes in seven different tissues. (**C**) The correlation of expression data for different genes. Red hues represent positive correlations, while blue hues represent negative correlations.

gene family has been documented in several horticultural crops such as Chinese cabbage, tomato, potato, and peas. Although the regulatory mechanisms of *BZR1* transcription factors have been well studied in *(A) thaliana* and potato, their characterization in *(B) oleracea* remains unexamined. Investigating these genes in *B. oleracea* is essential for understanding their evolutionary patterns and functional roles for deeper insights into their regulatory functions. A comprehensive genome-wide analysis of *BoBZR1* genes has been conducted, encompassing their identification, chromosomal localization, evolutionary relationships, structural organization, and gene duplication or loss events. Additionally, this study explored their functional aspects and expression patterns across specific tissues.

In the entire genome of *B. oleracea*, twelve *BZR1* family genes have been identified. Notably, Arabidopsis contains six *BZR* members[62]. Beyond the initially recognized *BZR* genes in Arabidopsis, research has revealed that *BAM7* & *BAM8* of the *BAM* family have DNA binding domain similar to that of BRASSINAZOLE RESISTANT1. This specific domain is a characteristic feature of transcription factors to maintain response with brassinosteroids[63]. The physicochemical elements of the *BoBZR1* proteins were carefully investigated. The molecular weights (Mw) of all *BZR1* proteins in *B. oleracea* were observed to fall within two distinct ranges: from 10.88 to 36.18 kDa and from 75.87 to 79.09 kDa. These proteins had a lower molecular weight than *BZR* gene families from other crops, such as cucumber. All identified *BZR1* proteins exhibited a conserved hydrophilic nature, indicating their potential role in water-associated cellular processes[64]. The placement of *BoBZR1* genes across the genome was discovered to be uneven, similar to that seen in Chinese cabbage. Furthermore, the subcellular localization of these genes revealed information about their prospective cellular activities, as proteins frequently execute jobs unique to their organelle of localization. Notably, 83% of *BoBZR1* genes were found in the nucleus, implying a primary involvement in transcriptional control, while the remaining 17% were found in the cytoplasm, indicating potential supplementary functional contributions outside the nucleus.

Comparative analysis of *BZR1* genes across different crops provides valuable insights into the evolutionary relationships within the *BoBZR1* gene family. Genes clustering within the same phylogenetic clade are likely to share similar functional roles, allowing predictions about the function of less-characterized genes based on their well-studied counterparts. To explore these evolutionary connections, a phylogenetic tree was constructed, revealing that *BoBZR1* genes were grouped into three distinct clades. Notably, one clade included *AtBZR1* (Arabidopsis), *BoBZR1* (*B. oleracea*), and *SlBZR1* (Tomato), suggesting that *BoBZR1* genes may exhibit functional similarities with their Arabidopsis and tomato homologs, given their close evolutionary association. Earlier studies have emphasized the significance of the exons-introns arrangement of a gene in the evolutionary process[65]. Through the structural and motifs analysis, it became clear that genes belonging to a similar clade and population exhibited similarities in terms of the numbers and positions of motifs and exons-introns[66]. Structural analysis of *BZR1* genes revealed that except of *BoBZR1-1*, *BoBZR1-3*, and *BoBZR1-4*, all the other genes share the

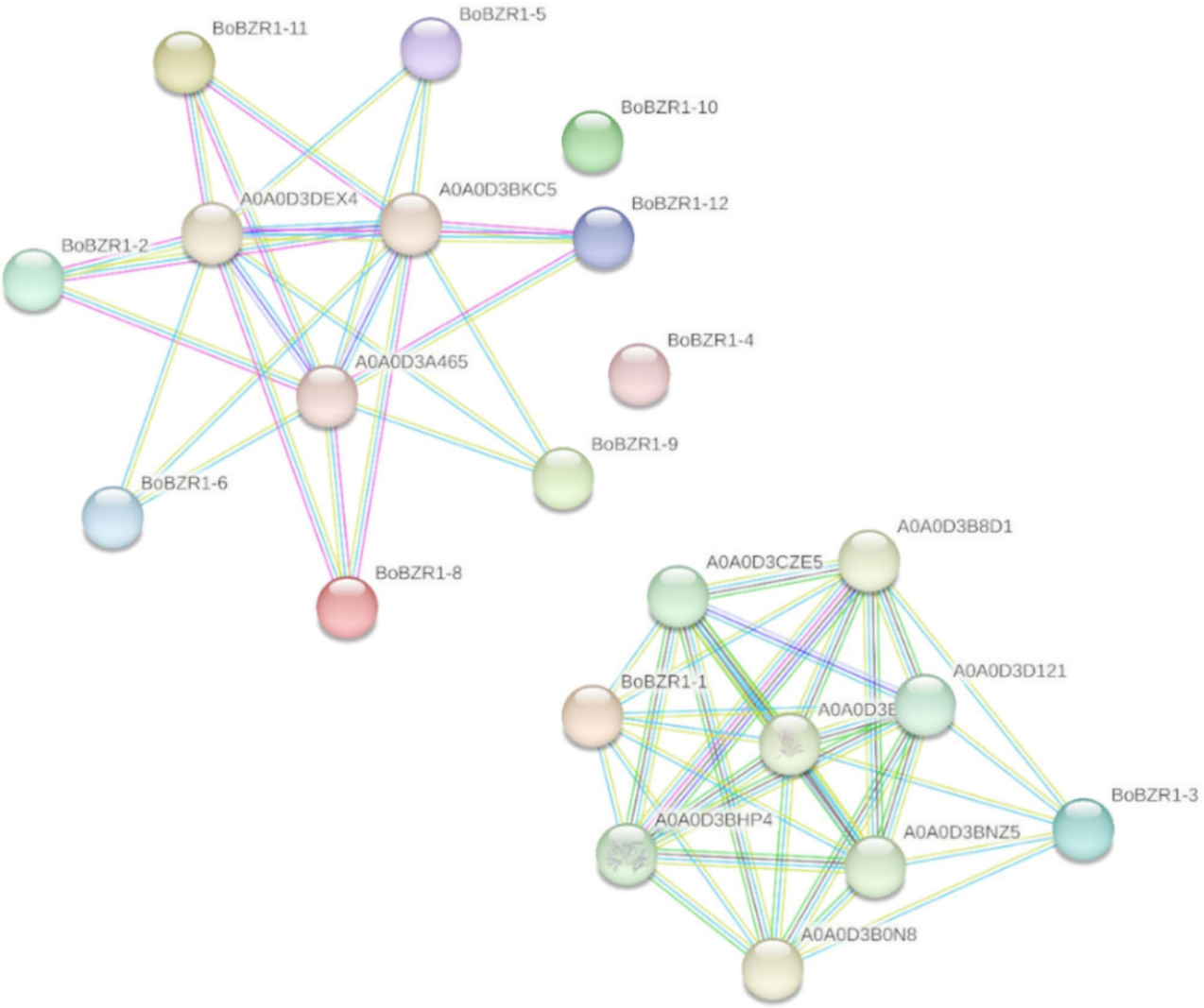

Fig. 8. Protein–protein interactions within the BZR1 genes of Brassica oleracea are represented by colored lines, indicating different types of interactions among the proteins. The identification of biosynthesis proteins is based on predictions from the STRING database.

same number of exons and introns. This observation suggests a potential conserved structural pattern among the majority of *BoBZR1* genes, highlighting the importance of structural features[67]. Furthermore, motif analysis revealed the presence of Motifs 1 and 3 in all *BoBZR1* genes, which was similarly detected in *CsBZR* and *AtBZR* genes. The conservation of these motifs implies a functional relationship between these genes, as such motifs frequently play an important role in gene regulation and activity. Their continuous existence across species demonstrates their importance in regulating gene function, probably contributes to the systemic regulation of *BoBZR1* genes.

The Brassica lineage is believed to have diverged from a common ancestor with Arabidopsis around 20 million years ago (Mya), followed by a Whole Genome Triplication event approximately 15.9 Mya, which led to extensive gene loss and rearrangement[68]. To explore the evolutionary history of *BoBZR1* genes, a comparative syntenic analysis was performed among *B. oleracea*, Arabidopsis, and potato, identifying conserved collinear regions. A notably higher number of *BoBZR1* gene copies were detected in Arabidopsis, suggesting a closer evolutionary relationship among these species. This indicates that these plants may have descended from a shared ancestor, with subsequent gene expansion and variation influenced by mutations and environmental adaptations, leading to species-specific differences in *BoBZR1* gene distribution. The identification of duplicated genes on the same chromosome implies a potential tandem duplication event, while the presence of duplicated genes on various chromosomes suggests a segmental duplication. The Ka/Ks ratio helps in understanding the evolutionary pressures acting on genes. A Ka/Ks value below 1 indicates purifying selection, which removes harmful mutations to preserve gene function. In *B. oleracea*, the analysis of duplicated *BoBZR1* genes showed Ka/Ks values below 1, suggesting they have mostly undergone purifying selection over time. Further examination of gene divergence and duplication events revealed variations in Ka/Ks ratios among different *BoBZR1* genes,

especially those that resulted from WGT. These differences indicate that *BoBZR1* genes have evolved under varying selection pressures, leading to different rates of evolution within *B. oleracea*[48].

Gene expression is crucial in the manifestation of functioning, and promoters play a direct role in influencing gene expression[69]. The promoter region of a gene includes specific elements that help regulate its activity in response to different factors. Among 12 *BoBZR1*, each member is associated with stress responsiveness, hormone responsiveness, and plant development responsive CREs. *BZR1* gene is likely to contain a significant role in governing growth and response to different kinds of stress, indicates the *BZR1* gene family serves as an essential element in the interacting BR signaling and other hormonal signals. Various reports confirmed the active participation of the *BZR* family in a diverse range of stress response mechanisms[21,70], hormone signaling[71], and the regulation of plant growth[72]. The analysis of *BoBZR1* genes in relation to cuticular wax development revealed that *BoBZR1-6*, *BoBZR1-7*, and *BoBZR1-10* showed over 1.2-fold higher expression compared to other genes. This increased expression may be associated with the presence of ABA-responsive cis-regulatory elements (CREs) in their promoter regions, suggesting their significant role in regulating growth and drought defense mechanisms. Similarly, in Arabidopsis, *BZR1* supports growth maintenance by binding to the BRRE box, highlighting its role in improving growth adaptation[73] and *BES1* plays a crucial regulatory role in cellulose biosynthesis by binding to the promoter regions of cellulose synthase genes, suggesting its involvement in stress adaptation and cell wall remodeling under environmental challenges[74]. In wheat, the transcription factor *TaBZR2*, belonging to the *BES/BZR* family, plays a key role in drought response by reducing the accumulation of reactive oxygen species through the activation of *TaGST*, a gene involved in detoxification and stress tolerance[75]. RNA-Seq analysis demonstrated that *BoBZR1* genes were consistently expressed across all examined tissues, indicating their fundamental role in cellular processes. This widespread expression suggests that BoBZR1 genes are key regulators in plant growth, development, and defense mechanisms, contributing to essential physiological functions throughout different stages of plant life[76]. The *BoBZR1* genes displayed widespread expression across all plant organs and were significantly regulated by developmental signals. Notably, *BoBZR1-6*, *5*, *9*, *2*, and *12* showed higher expression levels in leaves, while *BoBZR1-11*, *4*, *8*, *10*, *1*, and *3* were more predominantly expressed in stems. This organ-specific expression pattern suggests that *BoBZR1* proteins play essential roles in plant growth and development. Similarly, in maize, the expression of *ZmBES1/BZR1* genes varied under *ABA*, far-red, red, and dark light treatments, highlighting their involvement in adaptive responses to environmental stimuli[31]. Higher expression levels of *BoBZR1-5*, *BoBZR1-9*, *BoBZR1-11*, and *BoBZR1-12* were observed during both vegetative and reproductive growth stages, suggesting their possible involvement in later developmental processes Additionally, the observed expression correlations among these genes (Fig. 7c) suggest potential interactions of *BoBZR1* genes. These findings provide insights into the potential roles of *BoBZR1* genes, laying the groundwork for future studies on gene regulatory networks and their roles in stress responses.

Conclusion

This study identified twelve *BoBZR1* genes in *B. oleracea* through in-silico analysis and classified them into three subgroups based on their evolution. These genes were unevenly distributed across chromosomes, and their promoter regions contained regulatory elements associated with growth, stress responses, light signaling, and hormone regulation. Expression analysis showed that *BoBZR1-2* had consistently high expression across multiple tissues, indicating its potential role in plant growth and development. Additionally, the involvement of *BoBZR1* genes in cuticular wax biosynthesis suggests their contribution to plant defense against environmental stresses. These findings offer a foundation for future research to further explore the functional significance of *BoBZR1* genes in stress adaptation and development.

Data availability

The NCBI-GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi using acc=GSE130405 and GSE42891) contains the datasets analyzed for this work.

Received: 20 February 2025; Accepted: 21 April 2025
Published online: 03 May 2025

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Author contributions

MS and MAU: prepare the first draft of the manuscript. Bioinformatics analysis was handled by MAU, AW, and SA. MAU, MH, RG, MS, AW, SA, TA, and MR: write, revised the manuscript and provided comments during the writing and revised it. The authors read and approved the final manuscript.

Funding

This work has received no funding.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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