

Article

Genome-Wide Identification and Expression Analysis of the Pepper β -1,3-gucanase Gene Family in Response to *Phytophthora capsici* Stresses

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Abstract: Peppers are widely cultivated around the world, yet they suffer from infections caused by *Phytophthora capsici* fungi throughout the year, which severely impacts their yield. The β -1,3-glucanase gene has been shown in previous studies to significantly enhance plants' ability to cope with both biotic and abiotic stresses, including fungal infections. However, its function in peppers has not been reported. In this study, 80 *CaBG* genes were initially identified, before being filtered down to 54 *CaBGs* in peppers, and analyses conducted on the physicochemical properties, chromosomal localization, phylogenetic tree relationships, synteny, promoters, and gene expression levels of the *BG* gene family. The results indicated that these 54 *CaBG* genes are located on 12 chromosomes. Phylogenetic tree analysis classified the *CaBGs* into three subfamilies, α , β , and γ , each with its own specific functions, with the γ subfamily being associated with disease resistance in peppers. Synteny analysis showed that *CaBG* genes are relatively conserved and have not undergone extensive whole-genome duplication events. Promoter analysis indicated that *CaBGs* are induced by plant hormones and various external stressors. Transcriptomic and RT-qPCR analyses revealed that the *CaBG7* and *CaBG12* genes were significantly activated following infection by *P. capsici*, with the expression levels of these two genes being markedly higher in resistant plants compared to susceptible ones. Based on the phylogenetic tree and gene expression analysis, we propose that *CaBG7* and *CaBG12* may be key genes for disease resistance in chili peppers. This study provides a theoretical basis for breeding *P. capsici*-resistant chili peppers by molecular breeding methods.

Keywords: pepper; β -1,3-glucanase; *Phytophthora capsici*; resistant



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1. Introduction

Phytophthora belongs to the family *Pythiaceae*, there are more than 120 species, most of which are important plant pathogens [1]. *Phytophthora* root and collar rot, caused by *Phytophthora capsici* (*P. capsici*), is the most serious disease in pepper production [2,3], and it causes more than USD 100 million in losses worldwide annually [4]. *P. capsici* belongs to

oomycetes, and there are more differences with fungi in terms of trophic structure, genome composition, metabolic pathways, and reproduction, and common fungal inhibitors are basically ineffective against them [5]. At present, control of oomycete diseases caused by *P. capsici* is still a worldwide problem, and the production of chemical-based agents, resulting in pathogen resistance, pharmaceutical residues and environmental pollution, and other issues is increasingly prominent [6]. Therefore, it is particularly important to find key genes that can suppress *P. capsici* and to develop molecularly improved varieties resistant to *P. capsici* for pepper. The cell wall is a critical biological structure that interfaces with host cells and plays a pivotal role in the pathogenicity and virulence of invasive fungi. Composed primarily of β -1,3-glucanase, chitin, and mannoproteins, the cell wall's composition and the ratio of these components vary significantly across different fungal species, influencing their functional properties and interactions with host organisms [7]. β -1,3-glucanase is a polysaccharide formed through glycosidic linkages of glucose, and β -1,3-glucanase accounts for the majority of β -1,3-glucanases in fungal cell walls. β -1,3-glucanases are synthesized by plasma membrane-associated synthetases and are transported to the cell wall during synthesis [8].

β -1,3-glucanase belongs to the glycoside hydrolase family 17, which is the Pathogenesis related-2 (PR-2) protein [9]. The β -1,3-glucanase gene family has been extensively characterized across various species, with its functional roles partially elucidated through comprehensive research efforts [10–12]. It can hydrolyze the main component of the pathogen cell wall, β -1,3-glucanase, effectively inhibiting the growth of pathogens, and it serves as a crucial defense factor in the plant defense system [13]. β -1,3-glucanase can be expressed in plant–pathogen interactions to improve plant defenses [14,15]. In *Arabidopsis*, there are 50 *AtBGs*, all of which contain an N-terminal signal peptide and a glycosyl hydrolase family 17 (GH-17) domain, with some also containing a C-terminal domain X8 (also known as CBM43) [16]. In tomato (*Solanum lycopersicum*), a phylogenetic analysis identified 51 candidate β -1,3-glucanases that were distributed in three clusters (α , β , and γ), with evolutionary relations previously characterized in the model *Arabidopsis thaliana* [17]. Previous studies have shown that β -1,3-glucanase genes can be significantly expressed when plants are subjected to pathogenic bacteria or abiotic stresses, thereby increasing plant stress tolerance. For example, with a soybean gene *SGN1* encoding β -1,3-glucanase, the expression of *SGN1* was strongly induced by a variety of defense-related signals, such as treatment with H_2O_2 , wounding, or treatment with fungal elicitor prepared from *Phytophthora* spp, as well as inoculation with *Pseudomonas syringae* [18]. The transient overexpression of the sugarcane (*Saccharum officinarum*) β -1,3-glucanase gene *ScGluD2* in tobacco leaves induced the plant's defense response and enhanced its resistance to *Pseudomonas solanacearum* and *Botrytis cinerea* [19]. In vitro antifungal assays revealed that a wheat (*Triticum aestivum*) recombinant β -1,3-glucanase significantly inhibited the spore formation and hyphal morphology of common wheat grain fungi [20]. A β -1,3-glucanase gene (*PnGlu1*) from *Panax notoginseng* exhibits a significantly induced expression in response to treatments with methyl jasmonate, salicylic acid, ethylene, hydrogen peroxide, and *Fusarium solani* inoculation. Recombinant PnGlu1 protein demonstrates high inhibitory activity against the growth of *Fusarium solani* and *Fusarium oxysporum*. Additionally, tobacco plants overexpressing *PnGlu1* show strong resistance to *Fusarium solani* infection [21]. A novel β -1,3-glucanase, Gns6, was found to have significant antifungal activity against *Mycobacterium bovis* in rice [21].

Peppers, belonging to the *Solanaceae* family, are currently the most widely cultivated vegetable species in China [22]. With the expansion of pepper cultivation in China, the impact of *P. capsici* infestation on pepper crops has become increasingly severe. *P. capsici* seriously affects the growth and development of peppers by infecting its stems, leaves, and other parts, which can lead to widespread plant death and significant yield loss [2,4]. In

previous studies, extensive research has been conducted on the molecular mechanisms of pepper resistance to *P. capsici*, primarily focusing on signal transduction pathways following pathogen infection and biological control strategies [23–25]. Additionally, significant progress has been made in screening pepper varieties resistant to *P. capsici*, with researchers successfully identifying multiple elite disease-resistant cultivars, contributing greatly to reducing the damage caused by *P. capsici* and improving pepper yields [26,27]. However, the precise identification of key genes directly involved in the disease resistance process and the enhancement of pepper resistance through molecular breeding techniques remain critical objectives in current research. Therefore, identifying key genes involved in pepper resistance to *P. capsici* blight is crucial. In this study, we conducted a comprehensive genome-wide identification of the β -1,3-glucanase gene family in peppers, identifying a total of 54 β -1,3-glucanase genes. We performed bioinformatic analyses to characterize the family of β -1,3-glucanase genes and expression patterns under biotic stress induced by *P. capsica*, providing a theoretical basis for the selection and breeding of pepper varieties resistant to *P. capsici*.

2. Materials and Methods

2.1. Plant Materials

In our pre-laboratory studies, we identified several resistant and susceptible pepper varieties by direct infestation of pepper plants by *P. capsici* and molecular marker experiments. In this experiment, the tested chili pepper varieties include a high-resistant variety R (V06C1825) and a susceptible variety S (H15-077), bred by the Institute of Vegetables, Anhui Academy of Agricultural Sciences. The *P. capsici* fungus HFGL-1-24 was cultured and provided by the Institute of Plant Protection and Agro-Product Safety, Anhui Academy of Agricultural Sciences. Pepper plants were cultivated in a constant-temperature laboratory incubator (20–25 °C in 16 h (h)/8 h light/dark cycles with 60% relative humidity; light intensity: 20,000 lux; regular watering and fertilization).

2.2. Genome-Wide Characterization of the β -1,3-glucanase Gene Family in Peppers

The chili pepper Zunla-1_v3.0 genome, selected for this study, was obtained from <http://www.bioinformatics-lab.cn/PepperBase/> (accessed on 8 February 2025) [28]. The Arabidopsis genome was obtained from <https://www.arabidopsis.org/> (accessed on 8 February 2025), and the tomato genome annotation (ITAG2.4) was obtained from <https://solgenomics.net/> (accessed on 8 February 2025). The hidden Markov model (HMM) profile of the glycoside hydrolase family 17 domain (PF00332) was obtained from the Pfam website (<http://pfam.xfam.org/>, accessed on 8 February 2025) and was employed as a query to identify all possible β -1,3-glucanases using HMMER (V3.0) software (e-value $\leq 1 \times 10^{-10}$) [29]. The identification of conserved domains was performed using NCBI CDD tools (<https://www.ncbi.nlm.nih.gov/cdd>, accessed on 8 February 2025) and the InterPro database (<https://www.ebi.ac.uk/interpro/>, accessed on 8 February 2025) to identify the CaBG gene family members in pepper and name them based on their chromosomal positions.

Candidate proteins were further subjected to multiple sequence alignment to remove duplicates and incomplete protein sequences. The physicochemical properties of CaBG family members were analyzed using the online tool the ExPASy Proteomics Server (<http://expasy.org/>, accessed on 20 November 2024) [30]. Subcellular localization analysis of each gene family member was performed using the WoLF PSORT online tool [31]. The chromosome distribution map of the β -1,3-glucanase family genes in pepper was constructed using TBtools software v2.0 [32].

2.3. Phylogenetic Analysis of β -1,3-glucanase Family Genes from Pepper, Arabidopsis, and Tomato

To find out the evolutionary relationship of the β -1,3-glucanase gene family of chili peppers, we combined β -1,3-glucanase protein sequences of pepper, Arabidopsis, and tomato to construct a phylogenetic tree. A multiple sequence alignment of BG proteins was performed using ClustalX2.1 (Multiple Alignment Parameters, Gap Opening: 10.00, Gap Extension: 0.20). The phylogenetic tree was constructed with MEGA 7.0 using the neighbor-joining method and 1000 bootstrap replicates [33].

2.4. CaBGs Gene Motif and Structure Analysis

The genomic, CDS, and protein sequences of CaBGs were obtained from the pepper Zunla-1_v3.0 genome (<http://www.bioinformatics-lab.cn/PepperBase/>, accessed on 21 November 2024) [28]. The exon/intron analysis of CaBGs was conducted using GSDS 2.0 (<http://gsds.gao-lab.org/index.php>, accessed on 22 November 2024). The motif analysis of CaBGs was performed in MEME (<http://meme-suite.org/>, accessed on 25 November 2024), with parameters set as follows: default for advanced options and a maximum of 10 motifs.

2.5. Cis-Element and Synteny Analysis of CaBGs

MCScanX software (using built-in parameters) was employed to investigate the collinearity relationship among the BG gene families in pepper, Arabidopsis and tomato (CPU for BlastP: 2, E-value: 1×10^{-10} , Num of BlastHits: 5) [34]. The results were visualized using TBtools software v2.0 [32]. The cis-acting elements of the promoters of the pepper BG family genes were analyzed within the 2.0 Kb upstream sequences from the transcription start sites of the BG family genes using the online website PlantCare (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 8 February 2025).

2.6. Analysis of Expression Patterns in Roots and Stems of Pepper Plants Under *P. capsici* Biostress

The laboratory conducted a preliminary screening and selected a susceptible variety (H15-077) and a resistant variety (V06C1825). The root irrigation method was employed to inoculate the seedlings of the susceptible variety H15-077 and the resistant variety V06C1825 with *P. capsici* (HFGL-1-24). Twelve hours prior to inoculation, the substrate was thoroughly watered to achieve a saturated moisture state. A glass rod was used to create holes approximately 1 cm away from the pepper plants in the substrate, into which 5 mL of spore suspension was injected. Each experimental material consisted of 15 plants. Following inoculation, the plants were incubated at 28 °C in dark conditions with high humidity for 24 h, after which they were maintained under normal management conditions. After inoculating these two pepper varieties with *P. capsici*, stem tissues were collected at 0 days (1 h), 1 day, 3 days, and 5 days post-inoculation. The tissues were quickly frozen in liquid nitrogen, with three biological replicates for each variety and stage to eliminate errors between samples. RNA extraction and cDNA library construction were carried out by Beijing Biomarker Technologies Co., Ltd. (Beijing, China). The cDNA libraries were then sequenced using the Illumina (San Diego, CA, USA) high-throughput sequencing platform to obtain the raw data (NCBI accession number PRJNA1234221). The percentage of Q30 bases of each sample of clean data obtained after sequencing quality control was not less than 93.86%, and the comparison efficiency of reads of each sample with the reference genome ranged from 93.15% to 95.27%. In order to analyze the effect of *P. capsici* on the gene expression of CaBGs in pepper roots, we also obtained and analyzed publicly available raw transcriptome data (NCBI accession number: PRJNA960669) from the roots of the A198 pepper variety under *P. capsici* biotic stress [4]. After raw data filtering, sequencing error rate checking, and GC content distribution checking, we obtain clean reads for subsequent analysis. We used FPKM (Fragments Per Kilobase of transcript per

Million fragments mapped) as a measure of transcript or gene expression level [34]. A heatmap of their expression under *P. capsici* stress conditions in pepper was visualized using TBtools v2.0 [32].

2.7. RNA Extraction and cDNA Reverse Transcription

After inoculating the highly resistant variety R (V06C1825) and the susceptible variety S (H15-077) with *P. capsici*, stem tissues were collected from the uninfected pepper samples (pepper plants before infestation by *P. capsici*) as the control group (CK), as well as from the pepper plants at 0 day (1 h), 1 d, 3 d, and 5 d post-inoculation. Three biological replicates of samples from each stage were taken to eliminate errors between samples. All the collected samples were rapidly frozen in liquid nitrogen and then stored at -80°C for subsequent RNA extraction. Samples were hand-ground in liquid nitrogen using mortar and pestle before mRNA extraction. Total RNA was extracted using the TIANGEN RNApure kit (Tiangen, Beijing, China), following the manufacturer's instructions. Reverse transcription was performed using an HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme Biotech, Nanjing, China). Three replications of samples were performed for each stage.

2.8. RT-qPCR Analysis

All RT-qPCR primers were designed using Beacon Designer 7 software (Table S3). The RT-qPCR was performed with a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA), with three biological replicates for each sample. The relative gene expression levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method [35]. *CaACT* (Genebank: AY572427.1) was used as reference gene for resistant variety R (V06C1825) and the susceptible variety S (H15-077) pepper qPCR [36].

2.9. Data Collection and Statistical Analysis

Data are presented as means $\pm$ SE of at least three independent experiments. The experimental data were analyzed by one-way ANOVA using SPSS 25.0 software; multiple comparisons were performed using Duncan's method ($p < 0.05$). Statistical analyses were performed using GraphPad Prism 8.0 software.

3. Results

3.1. Identification and Physicochemical Characteristics of the β -1,3-glucanase (BG) Family Members in Pepper

This research identified 80 *CaBG* genes in the pepper genome through the use of the hidden Markov model PF00332. Candidate proteins were identified through conserved domain and subjected to multiple sequence alignment to remove duplicates and incomplete protein sequences, with 54 remaining after removing duplicates and short fragments. The *CaBG* proteins exhibited a variation in amino acid length, ranging from 195 amino acids (aas) in *CaBG6* to 583 aas in *CaBG37*, with corresponding molecular weights ranging from 22.03 kDa (*CaBG6*) to 63.17 kDa (*CaBG7*). The isoelectric points ranged from 4.62 (*CaBG54*) to 9.48 (*CaBG12*), and there were 40 *CaBG* proteins with isoelectric points less than 7, which accounted for the majority of the *CaBG* family, indicating that most of the proteins in the *CaBG* family were acidic proteins. The most predicted sites for the subcellular localization of *CaBG* proteins were chloroplast (chlo) and plasma membrane (plas), with 13 genes, followed by extracellular (extr) with 9 genes and then vacuolar membrane with 7 genes (Table S1).

3.2. Chromosome Localization of Pepper BG Genes

We conducted the chromosomal localization of 54 BG genes in pepper, revealing their distribution across all 12 chromosomes (Figure 1). Chromosome 3 exhibited the highest gene density, with ten BG genes identified. Chromosomes 1 and 5 followed closely, each harboring eight CaBGs. In contrast, chromosomes 6, 8, 9, and 10 contained the fewest CaBG genes, with only two genes localized on each. This uneven distribution suggests that chromosomes 1, 3, and 5 may play a pivotal role in the evolutionary dynamics of CaBG genes in pepper.

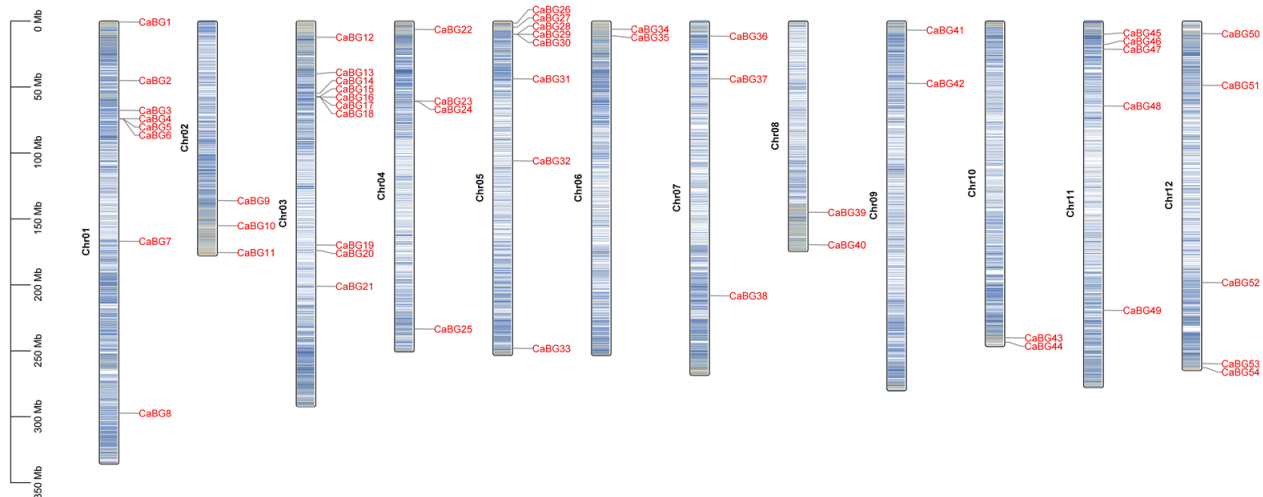


Figure 1. Distribution of the pepper BG family genes on the chromosomes.

A further analysis of gene positioning revealed the presence of gene clusters on specific chromosomes. These clusters, composed of genes with similar or related functions, likely contribute to coordinated biological roles within the organism. Notable examples include the clusters *CaBG4*, *CaBG5*, and *CaBG6* on chromosome 1; *CaBG14*, *CaBG15*, *CaBG16*, *CaBG17*, and *CaBG18* on chromosome 3; and *CaBG28*, *CaBG29*, and *CaBG30* on chromosome 5. The clustering of these genes underscores their potential functional synergy and evolutionary significance.

3.3. Phylogenetic Tree Analysis of CaBG Family Genes

To study the evolutionary relationship between *CaBGs*, *SIBGs* and *AtBGs*, a phylogenetic analysis was performed (Figure 2). The results showed that the 54 *CaBGs*, 50 *SIBGs* [17], and 50 *AtBGs* [16] were classified into three subfamilies (α , β , and γ), consistent with previous studies in Arabidopsis [16]. β -1,3-glucanases are considered key regulators responsible for plant pollen and fruit development, seed production, and disease resistance. To identify candidate pepper orthologues, we analyzed the branching pattern of the phylogenetic tree. The results showed that in the α subfamily, *CaBG31*, *10*, *8*, and *3* clustered together with the plasma membrane proteins AT3G13560.1 (PdBG1), AT2G01630.1 (PdBG2), and AT1G66250.1 (PdBG3) from the Arabidopsis β -1,3-glucanase family. The proteins AtPdBG1, 2, and 3 are associated with plasmodesmal conductivity and callose turnover [37]. In the Arabidopsis β -1,3-glucanase family, AT1G64760.1 (*ZET*) and AT2G19440.1 (*ZETH*) are homologous genes. Studies have shown that the *ZET* gene is related to plant growth and development, and silencing the *ZET* gene leads to abnormal development in Arabidopsis plants [38]. In the β subfamily, the *CaBG19*, *CaBG44*, and *CaBG52* genes cluster together with AT1G64760.1 (*ZET*) and AT2G19440.1 (*ZETH*), suggesting that these genes may share similar functions. In the γ subfamily, *Solyc11g065280.1* (*SIBG10*) is a homolog of *CaBG48*, with functions primarily related to callose deposition

in tomatoes and male sterility [39]. At4g16260.1, AT3G57270.1 (AtBG1), AT3G57260.1 (AtBG2), and AT3G57240.1 (AtBG3) are Arabidopsis virus defense proteins. Among them, At4g16260.1 shows significant expression responses to the highest number of pathogens (four species), and it is induced to the greatest extent under pathogen stress among all PR genes (induced 22-fold and 53-fold by *Alternaria brassicicola* and *Phytophthora infestans*) [16]. These genes primarily belong to the γ subfamily and show high homology with CaBG7, CaBG12, CaBG26, and CaBG43. We predict that CaBG7, CaBG12, CaBG26, and CaBG43 may be involved in the defense against pathogens in chili peppers.

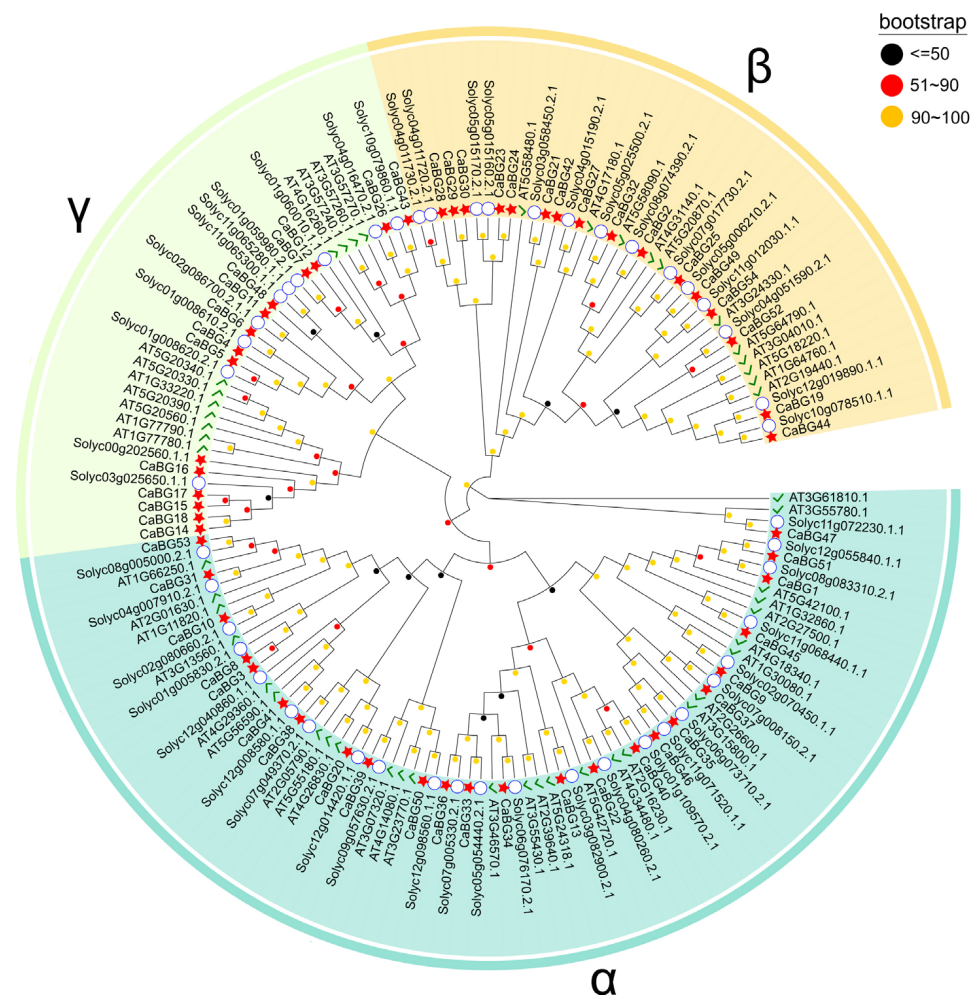


Figure 2. Phylogenetic relationships of pepper, tomato, and Arabidopsis BG proteins. The red star represents the protein in pepper. The white circle represents the protein in tomato. The green checkmark represents the protein in Arabidopsis.

3.4. Gene Structure and Conserved Motif Analysis of Pepper BG Genes

We conducted a phylogenetic tree construction using the protein sequences from 54 pepper BG genes and analyzed these genes for gene motif and gene structure (intron–exon). Among them, the α subfamily contained the largest number of CaBGs, with 24 members, followed by the β subfamily, with 16 members, and finally, the γ subfamily, which contained 14 CaBGs (Figure 3). The motif analysis showed that the CaBG proteins exhibited strong conservation in the distribution pattern and quantity of motifs. An analysis revealed that motifs 2 and 7 correspond to conserved sequences of the β -1,3-glucanase GH-17 domain. As shown in Figure 3, all CaBG proteins contain these two motifs. Most of the CaBG proteins (47 in total) contain 10 motifs (maximum number), with only a few CaBGs having fewer than 10 motifs. Among them, CaBG6 and CaBG48 have the fewest

motifs, with only seven motifs. An interesting observation is that the genes with fewer than 10 motifs are concentrated in the γ and β subfamilies, while the CaBG proteins in the α subfamily all possess the complete set of 10 motifs (Figure 3).

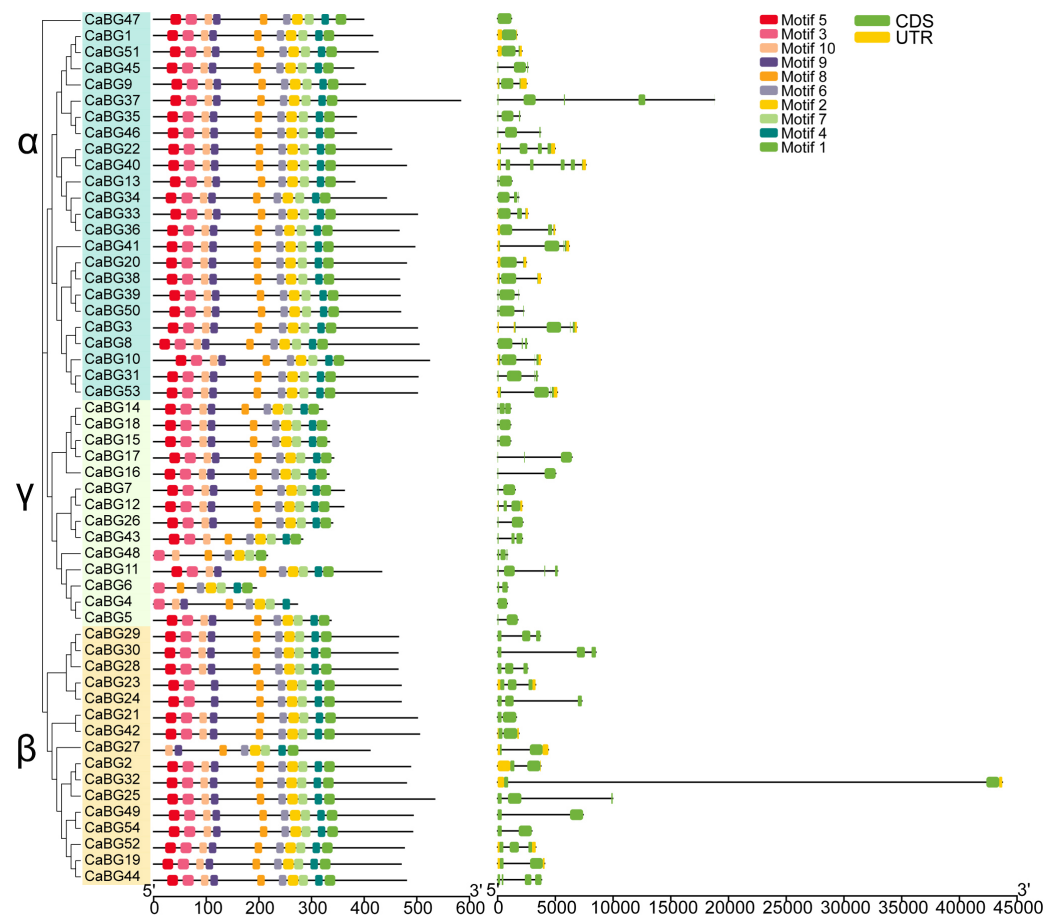


Figure 3. Gene structures and protein motifs of the pepper BG gene family.

In the context of multi-gene families, the structural disparities in exon–intron within the same family serve as pivotal factors in the evolutionary process. We observed the gene structure of the pepper BG gene family and found that the number of exons is generally low. The number of exons in most CaBG genes is predominantly concentrated in the range of one to four. Among them, *CaBG40* contains the most exons (six in total). Additionally, some genes lack intronic sequences, consisting of only a single exon, such as *CaBG4* and *CaBG47*. Genes within the same branching of the evolutionary tree exhibit similarities in their exon–intron structure (Figure 3). The resemblance in these gene structures, coupled with a high sequence homology, suggests that gene duplication events transpired in the evolution of the pepper BG gene family.

3.5. Synteny Analysis of BG Genes Among Pepper, Arabidopsis, and Tomato

To better understand the evolutionary relationships of BG gene families in pepper, Arabidopsis, and tomato, we conducted a synteny analysis within and between species of those BG genes (Figure 4, Table S2). The results showed that there are only 2 pairs of syntenic genes (*CaBG22/CaBG40* and *CaBG33/CaBG34*) within the pepper species (Figure 4A), which is significantly fewer compared to the 18 pairs between pepper and Arabidopsis (Figure 4B), and the 35 pairs between pepper and tomato (Figure 4C). This suggests that the pepper BG gene family is relatively conserved and has not undergone extensive whole-genome duplication events. The number of syntenic gene pairs between pepper and

tomato is significantly higher, with 35 pairs, compared to the 18 pairs between pepper and Arabidopsis. This difference can be attributed to the relatively short divergence time between species like pepper and tomato, which results in fewer accumulated variations and the preservation of more ancestral traits. In contrast, species with a longer divergence time, such as pepper and Arabidopsis, have accumulated more variations, leading to fewer shared traits and shorter collinear segments [40].

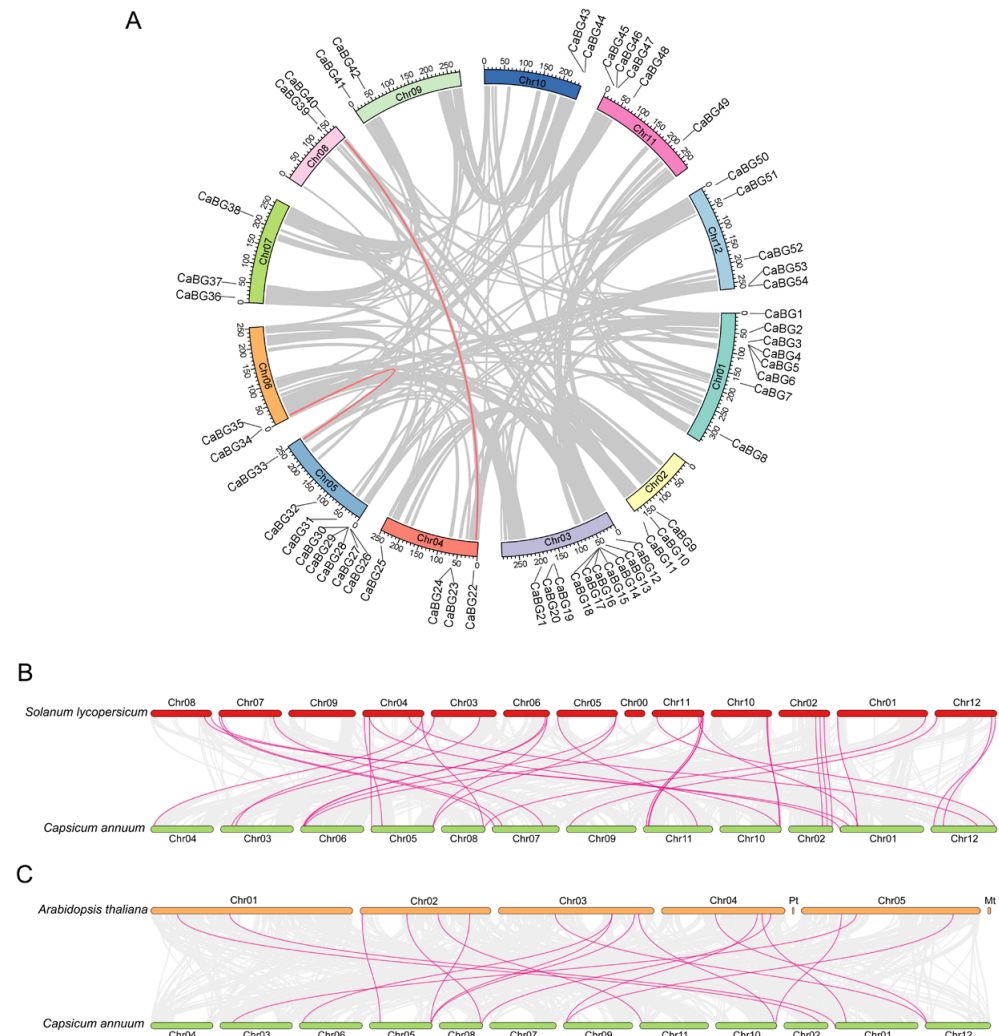


Figure 4. Analysis of synteny between pepper, Arabidopsis, and tomato. (A) Synteny between peppers. (B) Synteny between pepper and tomato. (C) Synteny between pepper and Arabidopsis. The gray lines represent the synteny relationships of the genes between pepper, Arabidopsis, and tomato. The pink lines represent the synteny relationships of the BG genes between pepper, Arabidopsis, and tomato.

3.6. Analysis of CaBG Promoter Cis-Elements

Through the analysis of cis-elements in the promoters, a total of 41 cis-elements were identified from the promoters of 54 *CaBGs*, and these elements were classified into 15 major categories (Figure 5). Among them, the transcription factor-related sites, such as MYB binding sites, accounted for the largest proportion (25.85%), followed by MYC binding sites (9.74%) and WRKY binding sites (2.97%). This indicates that *CaBGs* are regulated by transcription factors such as MYB, MYC, and WRKY, with MYB playing a dominant role in this regulation. In terms of environmental stress response elements, light response elements dominate, accounting for 24.09% of all elements, followed by defense and stress response elements (6.68%), low-temperature response elements (1.38%), and wound

response elements (1.5%). This suggests that the expression of *CaBGs* may be largely influenced by light induction. Hormone-responsive elements include those for MeJA, abscisic acid, salicylic acid, and auxin, with MeJA having the largest proportion (7.29%) and auxin the smallest (1.06%). Additionally, there were growth and development-related response elements, such as anaerobic induction (3.82%), meristem expression (1.12%), and endosperm expression (0.65%).

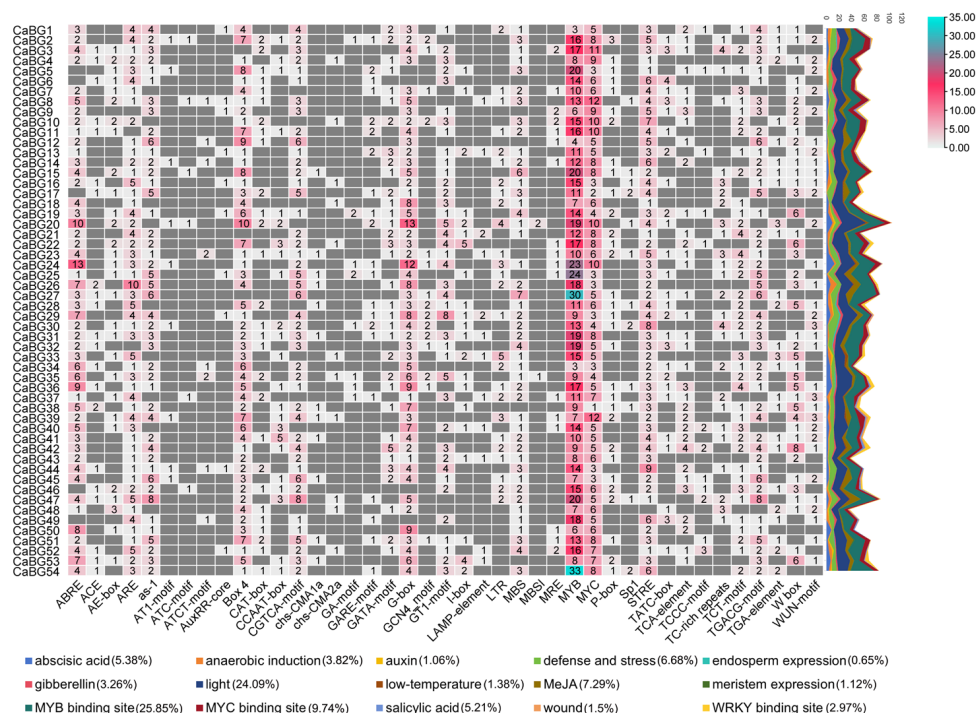


Figure 5. Cis-elements in the promoter region of pepper BG genes. The data in the heatmaps represent the number of cis-elements.

3.7. Expression Pattern Analysis of *CaBG* Genes Under *P. capsici* Infestans Stress

We conducted an expression analysis of the stems and roots of peppers after infection with *P. capsici*. Pepper stem samples were compared between the susceptible pepper (S) and the resistant pepper (R) (Figure 6A). In pepper roots, we compared the changes in the expression of *CaBGs* after infestation at different times (0 hpi, 24 hpi, 48 hpi) (Figure 6B). The findings indicate that in the stem, only three genes were significantly upregulated across all four time points when comparing S and R, namely *CaBG34*, *CaBG7*, and *CaBG12*. Among these, *CaBG34* generally exhibited a low expression level after infestation at different times; *CaBG7* and *CaBG12* expression was higher at 0 d (1 h) and 1 d of infestation, and then gradually decreased; and the expression of these genes was significantly higher in the disease-resistant variety R than in the susceptible variety S. In the roots, *CaBG7* and *CaBG12* were the two genes with the highest expression levels within the *CaBGs* family, and their expression increased with the duration of *P. capsici* infection at 12 and 24 h. From our previous evolutionary tree analysis, we found that disease resistance-related genes in Arabidopsis are primarily distributed in the γ subfamily. Both *CaBG7* and *CaBG12* belong to the γ subfamily and have a significant homology with Arabidopsis pathogen defense proteins *At4g16260.1*, *AT3G57270.1* (*AtBG1*), *AT3G57260.1* (*AtBG2*), and *AT3G57240.1* (*AtBG3*). Therefore, we speculate that *CaBG7* and *CaBG12* are key genes in chili pepper resistance against *P. capsici*. Interestingly, after infection, the expression level of *CaBG7* in the stem was lower than that of *CaBG12*, while its expression level in the roots was significantly higher than that of *CaBG12*. This suggests that *CaBG7* may function specifically in the roots, whereas *CaBG12* is more inclined to play a role in the stems.

3.8. RT-qPCR Analysis of the Pepper BG Gene Family

To validate the accuracy of the transcriptome data, we selected all the γ subfamily of *CaBG* genes from the pepper BG gene family (Figure 7). According to our RT-qPCR test results, the expression patterns were generally consistent for the transcriptome data we focused on. Among these genes, *CaBG6*, *CaBG14*, *CaBG16*, *CaBG17*, and *CaBG18* did not show any detectable expression levels. Additionally, except for *CaBG7* and *CaBG12*, the expression levels of the other genes were generally low and did not exhibit any clear patterns. The expression levels of *CaBG7* and *CaBG12* significantly increased after infection with *P. capsici*, with the expression in the R pepper plants being notably higher than in the S pepper plants. However, the expression levels of both R and S plants significantly decreased at 3 d and 5 d post-infection. This suggests that *CaBG7* and *CaBG12* are genes responsive to *P. capsici* infection, primarily acting in the early stages post-infection (0 d and 1 d), with their expression levels significantly declining at 3d and 5d after infection.

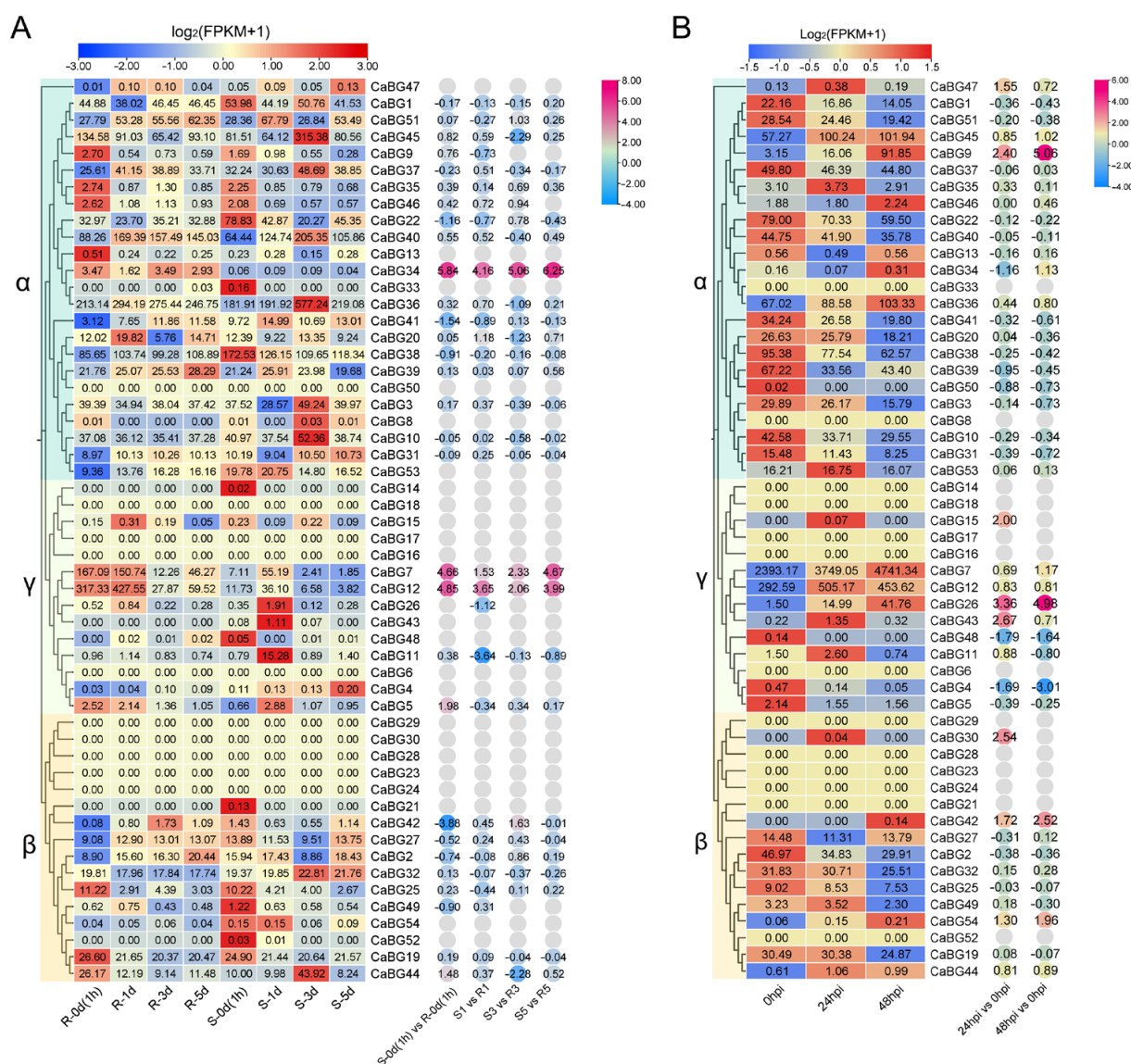


Figure 6. The expression patterns of pepper BG genes in response to *P. capsici* infestans stress. (A) Heatmap of *CaBG* gene expression in susceptible (S) and resistant (R) pepper stems under *P. capsici* infestans stress. (B) Heatmap of *CaBG* gene expression in pepper roots under *P. capsici* infestans stress. hpi: hour post-infection. The data in the heatmaps are the original FPKM values.

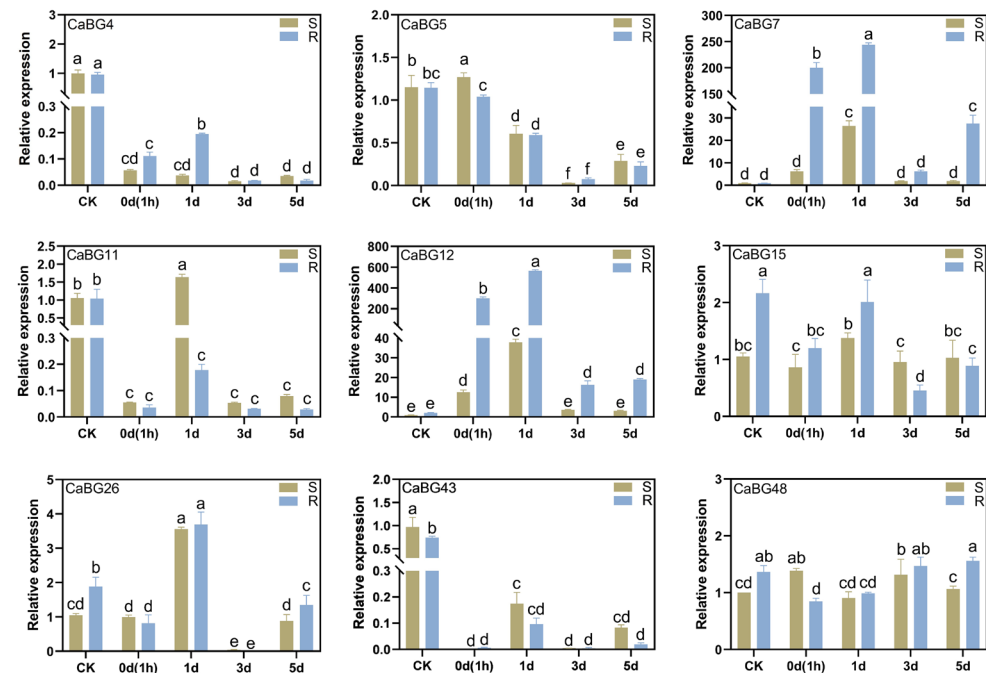


Figure 7. Relative expression level of pepper BG genes in response to *P. capsici* infestans stress. S: susceptible pepper, R: resistant pepper. Error bars are the average error of three biological replicates. Different lowercase letters in the data column indicate significant differences ($p < 0.05$) according to Duncan's test.

4. Discussion

During the growth and development of peppers, they are subjected to various biotic and abiotic stresses, making them vulnerable to multiple diseases [41,42]. Among these, *Phytophthora* blight, caused by *P. capsici*, is a significant threat to global pepper production [23,43]. This disease is particularly prevalent in hot (25–28 °C) and humid conditions, and in severe cases, it can lead to total yield loss [23,44]. *Phytophthora* blight has occurred in numerous countries and regions worldwide, and it is estimated that the economic losses caused by this disease exceed one billion dollars annually [45,46]. In our previous study, we screened pepper plants resistant to *P. capsici* and performed transcriptome sequencing on both resistant and susceptible plants, with the aim of identifying key genes involved in the resistance to this pathogen.

β -1,3-glucanase is plant PR-2 enzyme belonging to the glycosyl hydrolase family 17 (GH-17) [47]. It plays a significant role in growth and development, reproduction, and resistance to both biotic and abiotic stresses in plants [14]. In fungi, β -1,3-glucanase is a major component of the cell wall [48,49], and β -1,3-glucanase can be expressed in plant–pathogen interactions to inhibit the growth of pathogens by disrupting their cell walls and improving plant defenses [7]. With advancements in sequencing technologies, more genomic and transcriptomic data are being published, and it has become particularly important to utilize these data to identify the genes and their family members that need to be studied. To date, a complex and diverse BG gene family has been found in tomato [17], tobacco [50], soybean [51], Arabidopsis [16], rice [52], and cotton [53]. In this study, we applied a similar research approach and used a gap-free chili pepper genome, Zunla v3.0 [28], to identify the BG gene family in peppers. A total of 54 BG genes were identified. In this study, we classified the pepper BG genes into three subfamilies, α , β , and γ , based on previous research [16,17]. In Arabidopsis, genes that have been functionally validated within the α subfamily, such as *AtPdBG1*, *AtPdBG2*, and *AtPdBG3*, are primarily associated with plasmodesmal conductivity and callose turnover [37]. In the β subfamily,

AtZET and *AtZETH* are mainly associated with plant growth and development [38]. In the γ subfamily, *At4g16260.1*, *AT3G57270.1* (*AtBG1*), *AT3G57260.1* (*AtBG2*), and *AT3G57240.1* (*AtBG3*) are Arabidopsis virus defense proteins [16]. Each β -1,3-glucanase gene in these subfamilies appears to have its specific function. Since this study primarily focuses on the role of β -1,3-glucanase in chili pepper's resistance to *P. capsici*, our attention is specifically directed towards the β -1,3-glucanase genes in the γ subfamily of peppers.

Gene clusters are genomic regions composed of two or more adjacent and functionally related genes, typically generated through gene duplication events and retaining characteristics of their common ancestral gene. Genes within a cluster often encode functionally related enzymes, which can form multi-enzyme complexes that cooperatively catalyze key reactions in specific metabolic pathways [54]. This structure not only enhances the coordination of gene expression but may also improve the organism's adaptability to environmental stresses [55,56]. In this study, through chromosomal localization analysis, we identified multiple gene clusters within the pepper *CaBG* gene family, such as *CaBG4*, *CaBG5*, and *CaBG6* on chromosome 1; *CaBG14*, *CaBG15*, *CaBG16*, *CaBG17*, and *CaBG18* on chromosome 3; and *CaBG28*, *CaBG29*, and *CaBG30* on chromosome 5. The formation of these gene clusters may be closely related to the adaptive selection of pepper against diseases during its long-term evolution. From an evolutionary perspective, the formation of gene clusters may result from the combined effects of gene duplication and natural selection. Gene duplication events provide additional genetic material to the genome, while natural selection preserves gene combinations that enhance organismal adaptability. In the evolutionary history of pepper, diseases may have acted as a strong selective pressure, driving the formation of gene clusters through the duplication and recombination of disease resistance-related genes, such as *CaBG* genes. This structure not only facilitates coordinated gene expression but may also enhance pepper's resistance to diseases through gene dosage effects. Furthermore, genes within a cluster may further optimize their disease resistance functions through subfunctionalization or neofunctionalization.

Whole-genome duplication, also known as polyploidy accompanied by gene loss, has long been a significant driving force in the genomic evolution of animals, fungi, and other organisms, particularly plants [57,58]. As whole-genome duplication progresses, significant changes in genome structure often occur, such as chromosomal rearrangements, gene inversions, and gene losses. The large number of duplicated genes generated by polyploidy becomes an important source of genetic innovation [59]. In this study, comparative genomics and synteny analysis were conducted on pepper, Arabidopsis, and tomato. It was found that there are only 2 pairs of syntenic gene pairs within pepper, 18 pairs between pepper and Arabidopsis, and 35 pairs between pepper and tomato. This indicates that the *BG* gene family is relatively conserved and has not undergone extensive whole-genome duplication events, leading to only two pairs of syntenic genes within pepper. Additionally, the highest number of syntenic pairs between pepper and tomato suggests that the variation accumulated between the source species is relatively low, thus retaining more ancestral traits, further evidence for the conservation of the *BG* gene family.

Different types of cis-acting elements on the promoter reveal the potential functions and regulatory differences of genes. In this study, we identified not only defense and stress-related cis-acting elements in the promoter regions of *CaBGs* but also multiple hormone-responsive elements, including those for methyl jasmonate (MeJA), abscisic acid (ABA), salicylic acid (SA), and auxin (IAA). Previous studies have demonstrated that these hormones can activate the expression of β -1,3-glucanase genes, thereby enhancing plant resistance to pathogen infection. For instance, in tobacco, researchers fused the promoter region of the β -1,3-glucanase gene with the GUS gene and found that it could respond to Tobacco Mosaic Virus (TMV), salicylate, ethylene, et al. [60]. The promoter of *BjPR2*

in *Brassica juncea* was induced by fungi, hormones, and wounds [61], and the BG gene promoter of sesame (*Sesamum indicum* L.) induced by ABA and drought [62]. We also found numerous light-responsive elements and binding sites for transcription factors such as MYB and MYC. These findings suggest that β -1,3-glucanase is not only activated by hormones but also influenced by photoperiods and regulated by transcription factors like MYB and MYC.

Previous studies have shown that the activity of β -1,3-glucanase can be upregulated in response to pathogens or abiotic stress, thereby enhancing the plant's stress tolerance [63,64]. For example, in strawberry (*Fragaria ananassa*), the expression level of the β -1,3-glucanase gene *FaBG2-1* increased 116.9-fold 48 h after infection with *Colletotrichum fragariae* [65]. The transcription level of the β -1,3-glucanase gene *TaGlu* in wheat increased 35-fold 24 h after inoculation with *Puccinia striiformis* [66]. *ScGluD2* has been identified as the β -1,3-glucanase gene in sugarcane, and its expression was upregulated 14.77-fold in the disease-resistant sugarcane variety 1d after treatment with *Sporisorium scitamineum*. Moreover, the expression level of *ScGluD2* was higher in the disease-resistant variety than in the susceptible variety during the early stages of interaction with the smut fungus (1 d or 3 d) [19]. This study also obtained similar results through transcriptome data analysis. By analyzing the expression levels of genes in the stems of both disease-resistant and susceptible pepper varieties, we found that the expression levels of the two genes, *CaBG7* and *CaB12*, in the disease-resistant plants were significantly higher than those in the susceptible plants at 0 days (1 h), 1 d, 3 d, and 5 d after *P. capsici* inoculation. In addition, the expression of *CaBG7* and *CaB12* peaked at 1 d post-inoculation but declined after 2 days. RT-qPCR results also showed that the expression of peppers in both S and R varieties showed an increasing and then decreasing trend at different times after infestation with *P. capsici*, and compared to the susceptible pepper variety, the expression levels of *CaBG7* and *CaB12* in the resistant-variety pepper stems significantly increased after infection. CaBGs act as proteins that hydrolyze the cell wall of *P. capsica*, and the reason for the decrease in its expression after 2 d of infestation deserves to be studied and explored in the future; it is possible that it also plays an important role in early pathogen signaling. Additionally, the data indicated that *CaBG7* and *CaB12* levels were also dramatically elevated in the roots of pepper plants following *P. capsici* infection. Phylogenetic analysis revealed that *CaBG7* and *CaBG12* share a high homology with the disease resistance genes *At4g16260.1*, *AT3G57270.1* (*AtBG1*), *AT3G57260.1* (*AtBG2*), and *AT3G57240.1* (*AtBG3*) in *Arabidopsis thaliana* [16]. Therefore, we speculate that *CaBG7* and *CaB12* are key genes involved in pepper resistance to *P. capsici*. Our subsequent work will focus on functionally validating these two genes, *CaBG7* and *CaB12*.

5. Conclusions

In this study, we identified 54 BG genes in the pepper genome through whole-genome analysis, and these genes are located on 12 chromosomes of pepper. A phylogenetic analysis of the BG gene families from pepper, tomato, and *Arabidopsis* categorized the BG gene family into three subfamilies (α , β , and γ). The disease-resistant genes we focused on are located in the γ subfamily, and the disease resistance genes in *Arabidopsis* show a high degree of homology with *CaBG7*, *CaBG12*, *CaBG26*, and *CaBG43* in pepper. Synteny analysis revealed synteny within pepper/pepper, with only two pairs of CaBG genes showing synteny. In contrast, there are 18 pairs of syntenic genes between pepper/*Arabidopsis* and 35 pairs between pepper/tomato. Promoter analysis indicates that CaBG genes are not only induced by hormones and environmental stress but may also be regulated by transcription factors such as MYB, MYC, and WRKY. Transcriptomic data showed that *CaBG7* and *CaBG12* are significantly activated in pepper stems after infection with *P. capsici*, and their expression levels are significantly higher in disease-resistant plants compared to

susceptible ones. This result was further validated by RT-PCR experiments. In pepper roots, the expression of *CaBG7* and *CaBG12* also increased significantly after *P. capsici* infection. In summary, these findings provide target genes for further validation of the function of pepper *CaBGs* in resistance to *P. capsici* and lay the foundation for the molecular breeding of pepper resistance to *P. capsici*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy15040802/s1>, Figure S1: Reference gene stability by NormFinder; Figure S2: Melting curve analysis of each gene in RT-qPCR; Table S1: Information and physicochemical properties of 54 *CaBG* family members; Table S2: Analysis of synteny between pepper, Arabidopsis, and tomato; Table S3: Primers used for *CaBG* gene RT-qPCR analysis.

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Abbreviations

The following abbreviations are used in this manuscript:

BG	β -1,3-glucanase
<i>P. capsici</i>	<i>Phytophthora capsici</i>
R	resistant
S	susceptible
D	day
hpi	hour post-infection

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