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Identification of PAL Gene in Purple Cabbage and Functional Analysis Related to Anthocyanin Synthesis

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Abstract: Anthocyanin is a characteristic nutrient of purple cabbage, and phenylalanine ammonia-lyase (PAL) is the rate-limiting enzyme for the synthesis of anthocyanin by the phenylpropane pathway, which is an important part of plant secondary metabolism. In this research, 7 *BrPAL*, 8 *BoPAL*, and 15 *BnPAL* genes from genomes of *Brassica rapa*, *Brassica oleracea*, and *Brassica napus*, divided into four subgroups, evolved from 4 *PAL* genes in *Arabidopsis*. The amplification and evolution of the *BrPAL* gene are due to segmental duplication and purifying selection. *BrPAL* genes clustered in the same clade have similar intron/exon structures and motifs. The cis-regulatory elements are divided into four categories: light, growth and development, stress and hormones. The qRT-PCR assays showed that most *BrPAL* genes were upregulated by UVA, low temperature and MeJA and downregulated by FR, high temperature, salt, PEG, IAA, ABA and GA, and there was a positive correlation between anthocyanin content and gene expression. This study can be used as a source for the function of the cabbage *PAL* gene and its molecular mechanism of regulating anthocyanin synthesis and provides a theoretical basis for the molecular breeding of cabbage.

Keywords: phenylalanine ammonia-lyase (PAL); purple cabbage; anthocyanin; qRT-PCR

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1. Introduction

Phenylalanine ammonia-lyase (PAL) affects the accumulation of secondary metabolites, such as lignin, flavonoids and hydroxycinnamic acid amide (HCAA) in plants by regulating the rate of phenylalanine entering the phenylpropanoid metabolic pathway [1]. It is a key enzyme and rate-limiting enzyme that connects primary metabolism and phenylpropanoid metabolism and catalyzes the first step of phenylpropanoid metabolism [2]. PAL gene was first reported in barley in 1961 [3], usually in the form of the gene family in plants, from several members of many species, such as four members in willow [4], five members in poplar [5], three members in lotus [6], and seven members in cucumber [7] to more than a dozen members in potato [8] and tomato [9]. The PAL gene contains the characteristics of the Lyase aromatic conserved domain [10]. This domain can change the chromatin structure and regulate the expression of genes in different developmental tissues of plants to participate in the regulation of phenylpropanoid metabolic pathways throughout the growth cycle [11]. It plays an important regulatory role in plant growth and development, pest resistance and stress resistance [12].

Four members of the *Arabidopsis PAL* family were identified [13], and it was shown that *AtPAL1* and *AtPAL2* are functionally specific in abiotic environments triggering flavonoid synthesis [14], *AtPAL1* and *AtPAL2* double-knockout mutants exhibit enhanced drought tolerance, greater sensitivity to UV-B light and impaired production of flavonoids, such as anthocyanins [15]. *CsPAL* is involved in anthocyanin synthesis through the regulation of the transcription factors *CsMYB* and *CsbHLHv*, where the expression of *CsPAL4* in shoots

showed a highly significant positive correlation with anthocyanin content in purple-leaved tea plants [11]. *VvPAL1* and *VvPAL5* were found to be involved in anthocyanin biosynthesis in white and red grapes [16]. *TaPAL32* and *TaPAL42* gene-silenced plants showed a higher disease severity than control plants 14 days after wheat stripe rust [17]. In potatoes, most *StPALs* are involved in response to high temperatures and drought [10]. *CiPALs* are differentially expressed during pecan seed, female flower, and graft-binding development and during drought stress [18]. These data suggest that members of the *PAL* gene family are functionally specialized in the synthesis of flavonoids, such as anthocyanins and can respond to a variety of environmental stresses; therefore, it is important to understand how different family members are regulated to confer their functions.

Chinese cabbage (*Brassica rapa* L. ssp. *Chinensis*), native to China, is a genus of *Brassica* in the cruciferous family. In recent years, there have been many purple subspecies or varieties of cabbage, such as purple Tsai-tai, purple pakchoi, and purple turnips. The pigments that appear purple in cabbage have been identified as anthocyanosides, a class of secondary metabolites in plants that belong to the flavonoid class of phenolic compounds [19]. Due to its bright color and richness in phytochemicals, such as thioglucosides, phenolic acids, carotenoids, and flavonoids [20], it exhibits antioxidant and anticancer effects with high nutritional value and is one of the most important sources of dietary intake of flavonoid [21,22].

Since anthocyanin production in plants is usually expressed in a tissue-specific manner and induced by adversity, the analysis of the specific evolutionary regulatory mechanisms of *PAL* family genes in purple cabbage would provide a basis for the mechanism of anthocyanin synthesis in cabbage. However, the regulation of the *PAL* genes in anthocyanin biosynthesis in cabbage lacks in-depth and systematic studies. Therefore, the identification and evolutionary analysis of the *BrPAL* family members and their characteristics using bioinformatics methods to explore their expression patterns in response to light abiotic stress, phytohormonal aspects, and the relationship with anthocyanin synthesis provide significant theoretical support for the investigation of the function of the *BrPAL* gene and offer a fresh perspective on the breeding direction for high yield and quality in cabbage.

2. Materials and Methods

2.1. Identification of the PAL Gene Family in Chinese Cabbage

The Chinese cabbage genome data (*Brassica rapa* annotated genome V3.5) [23], *Brassica oleracea* genome database, *Brassica napus* genome database from the *Brassica* database (http://brassicadb.cn, accessed on 5 July 2022), and *Arabidopsis* database (https://www.arabidopsis.org/, accessed on 5 July 2022) were downloaded. Using BLASTP, the protein sequences of four *AtPAL* proteins identified in *Arabidopsis* were compared with those of Chinese cabbage to screen candidate genes. At the same time, the hidden Markov model (HMM) of the *PAL* gene-specific lyase aromatic domain (PF00221) came from the Pfam database and downloaded (http://pfam.sanger.ac.uk/ accessed on 5 July 2022) [24] using HMMER3.0 [25] software to screen *BrPAL* family members from the Chinese cabbage genome database, and the screening criterion was an E-value $\leq 1 \times 10^{-5}$. The redundant sequences between HMMsearch and BLASTP were removed, and candidate members were submitted to the online site SMART (http://smart.embl-heidelberg.de/, accessed on 6 July 2022) and the NCBI Conserved Domain Database CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd, accessed on 6 July 2022) [26] to verify the integrity of the conserved domain of the candidate gene protein and finally obtain the *BrPAL* family members.

2.2. Physicochemical Analysis and Subcellular Localization Prediction of PAL Gene Family Member Proteins in Chinese Cabbage

The physical and chemical properties of *BrPAL*, including the number of amino acids (aa), isoelectric point (pI), molecular weight (MW), and hydrophilicity and hydrophobicity (GRAVY), were analyzed using the ExPASy online program (https://web.expasy.org/protparam/, accessed on 10 July 2022). The Plant-mPLoc online tool (http://www.csbio.

sjtu.edu.cn/cgi-bin/PlantmPLoc.cgi, accessed on 12 July 2022) was used to predict the subcellular localization information of the *BrPAL* protein.

2.3. Phylogenetic Analysis of the Cabbage PAL Gene

Using MEGA 7.0 to retrieve protein sequences from *Arabidopsis*, *Brassica oleracea* and *Brassica napus PAL* to construct a phylogenetic tree, the sequences were multiply aligned, and the phylogenetic tree was constructed using the neighbor-joining (NJ) and maximum likelihood (ML) methods. The parameters were set as Poisson correction, pairwise deletion and bootstrap test (1000 repetitions), and the ITOL online website (http://itol.embl.de, accessed on 23 July 2022) was used to beautify and adjust the phylogenetic tree according to the phylogenetic relationship classification of the *PAL* gene family.

2.4. Chromosomal Mapping and Synteny Analysis of PAL Gene Family Members in Chinese Cabbage

The GFF3 file of the Chinese cabbage genome was utilized to extract the chromosome number, length, and *PAL* gene family member start and stop positions on chromosomes, and the MapGene2Chromosome online website (V.2.0; http://mg2c.iask.in/mg2c_v2.0/, accessed on 3 June 2022) was used to map the distribution of *PAL* gene family members on chromosomes.

MCScanX [27] software was used to perform a BLAST comparative analysis of the tandem and segmental duplication events in the Chinese cabbage genome, and TBtools [28] with homology visualization was used to compare Chinese cabbage with *Arabidopsis*, *Brassica napus*, and cabbage to visualize the collinear relationship. In addition, TBtools with a simple Ka/Ks(S2) calculator were applied to calculate Ka/Ks values between gene pairs.

2.5. Conserved Motifs and Gene Structure Prediction of the PAL Gene Family in Chinese Cabbage

The gff3 annotation file of Chinese cabbage was downloaded from the *Brassica* database (BRAD, http://brassicadb.cn, accessed on 5 July 2022), and the GSDS (http://gsds.gao-lab.org/, accessed on 8 July 2022) [29] online tool was used to analyze the members of the Chinese cabbage *PAL* gene family. The gene structure was visualized and analyzed, and a conserved domain analysis was performed using MEME (http://meme-suite.org/tools/meme, accessed on 7 July 2022) online software. The maximum number of motifs was set to 18, and the other parameters were the default parameters.

2.6. Analysis of Cis-Acting Elements in the Promoter of the PAL Gene Family in Chinese Cabbage

To discover the important cis-elements inside the *BrPAL* gene promoter, the 2000 bp sequence upstream of the translation start site of the *BrPAL* gene was extracted using TBtools through PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 5 June 2022) to predict the cis-acting promoter elements, and TBtools was used to construct the heatmaps and visualize them after the statistical screening.

2.7. Growth and Treatment of Cabbage Seedlings

To analyze the relationship between the *PAL* gene family of Chinese cabbage and its response to abiotic stress and phytohormones, "Jingyan" purple fast cabbage was hydroponically cultured till the three-leaf stage under normal conditions (25/18 °C, 75% relative humidity, and 12/12 days/nights). The light treatments were as follows: a light quality treatment with white LED light as the control (CK), with white light plus 10 μmol m^{-2} s^{-1} UV-A (UV-A); white light plus 60 μmol m^{-2} s^{-1} FR (FR); and white light plus UV-A and FR (FU). The total photon flux density (PPFD) of the different light treatments was 250 μmol·m^{-2}·s^{-1} for 10 days. For abiotic stress, a low-temperature/high-temperature treatment (4 °C/38 °C), drought treatment (10% PEG 6000) and NaCl treatment (200 mmol L^{-1}) was used; for the hormone treatment, auxin (100 μmol L^{-1} IAA), abscisic acid (100 μmol L^{-1} ABA), methyl jasmonate (100 μmol L^{-1} MeJA) and gibberellin (100 μmol L^{-1} GA3) were used. The samples were collected after 10 days of light quality treatment and after 3, 6, 12 and 24 h of

stress and hormone treatment. The collected samples were immediately placed in liquid nitrogen and stored at −80°C. Three biological replicates were performed for each sample.

2.8. Quantitative Real-Time Fluorescent Quantitative PCR and Anthocyanin Content Determination and Data Analysis

The total RNA of all samples was extracted using the Vazyme FastPure® Plant Total RNA Isolation Kit (Polysaccharides & Polyphenolics-rich), and the integrity and concentration of RNA were detected by 1% agarose gel electrophoresis and NanoDrop ND1000 (Nanodrop, USA). The RT-qPCR cDNA was synthesized using the TIANGEN FastKing One-Step Genomic cDNA First-Strand Synthesis Premix Kit and stored at −20 °C after ten-fold dilution. RT-qPCR primers (Supplementary Materials Table S1) were designed using Premier 5.0 and submitted to NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 3 August 2022) for a specificity check. The RT-qPCR was performed according to the SYBR® Premixm Ex Taq™ (Takara Biomedical Technology Beijing) reagent instructions and detected on a LightCycler® 96 Real-Time PCR instrument (Roche, Basel, Switzerland). Each sample had three biological replicates and three technical replicates according to the Ct value using the $2^{-\Delta\Delta CT}$ method [30]. The pH differential method was used to determine the anthocyanin concentration. SPSS (IBM® SPSS® Statistics version 24, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) were used for the data processing and graphing.

3. Results

3.1. Analysis of Chromosomal Location and Protein Physicochemical Properties of PAL Gene Family Members in Chinese Cabbage

A total of seven members of the *PAL* gene family were identified in the cabbage genome, and they were unevenly distributed on 4 of the ten chromosomes of Chinese cabbage (as shown in Figure 1). There were three genes on the A04 chromosome and two genes on the A05 chromosome. Chromosomes A07 and A09 each had one gene, which was distributed at both ends of the chromosome, except *BrPAL2*, and were renamed *BrPAL1-7* according to their position on the chromosome.

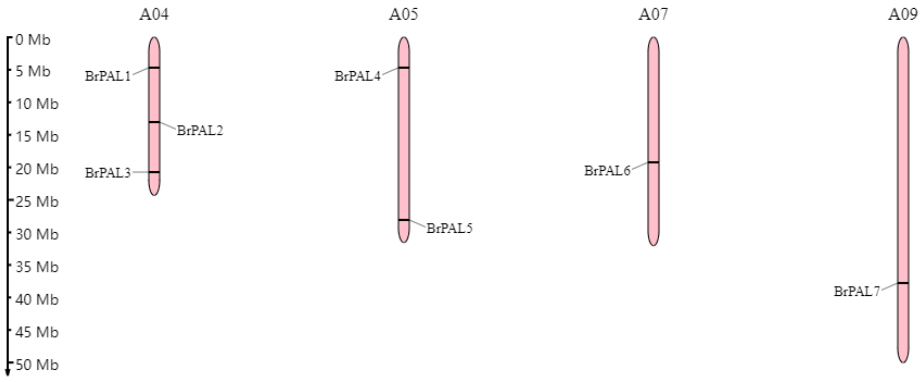

Figure 1. Chromosomal locations of seven *BrPALs* in the cabbage genome.

An analysis of the physicochemical properties of the seven identified *PAL* gene family members in Chinese cabbage (Table 1) showed that the number of amino acids in *BrPAL* ranged from 587 to 724 aa, the relative molecular weight was 64.304 to 78.524 kD, the isoelectric points were between 5.9 and 6.23, they were acidic proteins, and the hydrophilicity of all *BrPAL* proteins was negative, indicating that the proteins in this family are all hydrophilic proteins. The analysis of the subcellular localization of the proteins shows that the *BrPAL* proteins are all located in the cytoplasm.

Table 1. Sequence characteristics of *BrPAL*.

Gene Name	Gene ID	Number of Amino Acids (aa)	Molecular Weight (MW/kD)	pI	GRAVY	Subcellular Location
BrPAL1	BraA04g006770.3.5C.1	724	78.52	6.03	−0.177	Cytoplasm
BrPAL2	BraA04g016310.3.5C.1	698	76.91	6.23	−0.168	Cytoplasm
BrPAL3	BraA04g027460.3.5C.1	722	78.30	5.9	−0.163	Cytoplasm
BrPAL4	BraA05g008230.3.5C.1	719	78.10	5.9	−0.136	Cytoplasm
BrPAL5	BraA05g037490.3.5C.1	706	76.88	5.69	−0.139	Cytoplasm
BrPAL6	BraA07g021930.3.5C.1	587	64.30	5.93	−0.164	Cytoplasm
BrPAL7	BraA09g046240.3.5C.1	723	78.52	5.97	−0.167	Cytoplasm

3.2. Phylogenetic Analysis of PAL Gene in Chinese Cabbage

To gain a deeper comprehension of the evolutionary link between *PAL* genes in Chinese cabbage, *Arabidopsis*, *Brassica napus*, and *Brassica oleracea*, based on the multiple sequence alignments of 7 Chinese cabbage, 4 *Arabidopsis*, 15 *Brassica napus*, and 8 *Brassica oleracea PAL* genes, a phylogenetic tree of the conserved domains of *PAL* was constructed using the neighbor-joining (NJ) method (Figure 2). The tree shows that the 34 *PAL* genes can be divided into four subgroups (I, II, III, and IV). Group IV is the largest group, containing 15 members, among which three belong to Chinese cabbage, seven belong to *Brassica napus*, four belong to *Brassica oleracea*, and one belongs to *Arabidopsis*. Each subgroup contains one of the four *Arabidopsis PAL* genes, indicating that *BrPAL3*, *BrPAL4*, and AT2G37040.1; *BrPAL2* and AT5G04230.2; *BrPAL5* and AT3G10340.1; and *BrPAL1*, *BrPAL6*, *BrPAL7*, and AT3G53260.1 have a close evolutionary relationship, suggesting that the *Brassica PAL* gene may have evolved from the *Arabidopsis PAL* gene. We can better understand the probable biological activities of the *BrPAL* family by considering how often members of the same subfamily may perform comparable tasks.

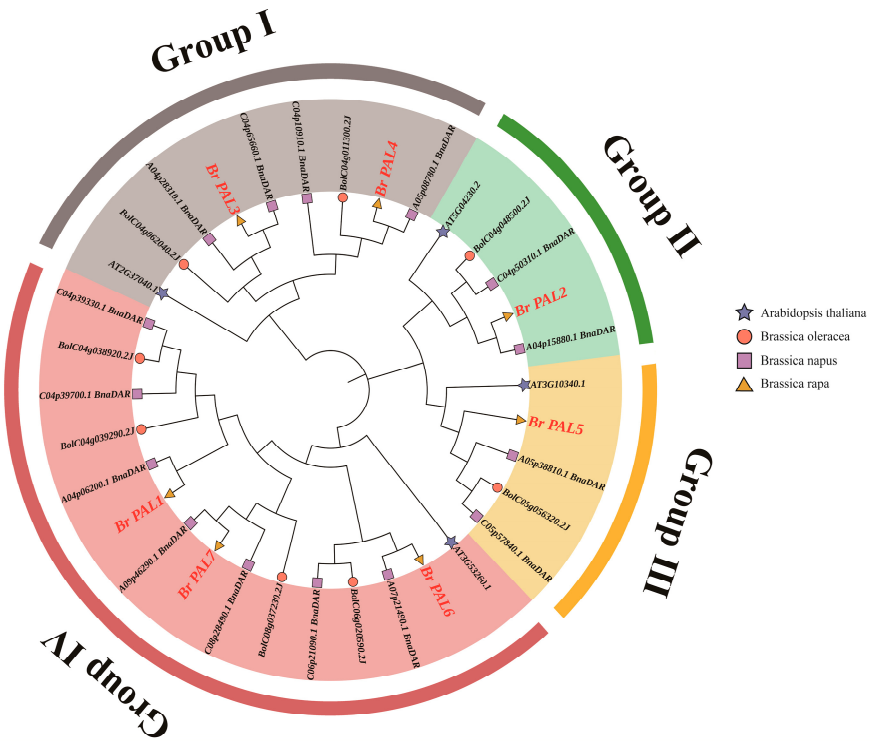

Figure 2. Phylogenetic tree of *PAL* from *Brassica rapa* (triangle), *Arabidopsis* (pentagram), *Brassica oleracea* (circle), and *Brassica napus* (square).

3.3. Collinearity Analysis of PAL Genes in Chinese Cabbage

Tandem and segmental duplications of genes are ubiquitous in plant genomes. As shown in Figure 3, no tandem duplication events occurred in the seven genes identified in this study, but four collinear gene pairs had segmental duplication events in the remaining five genes except *BrPAL2* and *BrPAL5*, indicating that segment duplication events are the main driving force of *PAL* gene diversity in Chinese cabbage. To explore the evolutionary restriction of *PAL* genes in Chinese cabbage, the ratio of the nonsynonymous mutation rate to the synonymous mutation rate (Ka/Ks) was calculated for each gene pair, and the results showed that the Ka/Ks of all collinear gene pairs was <1; this implies that the cabbage *PAL* gene family may have undergone strong purifying selection pressure during evolution.

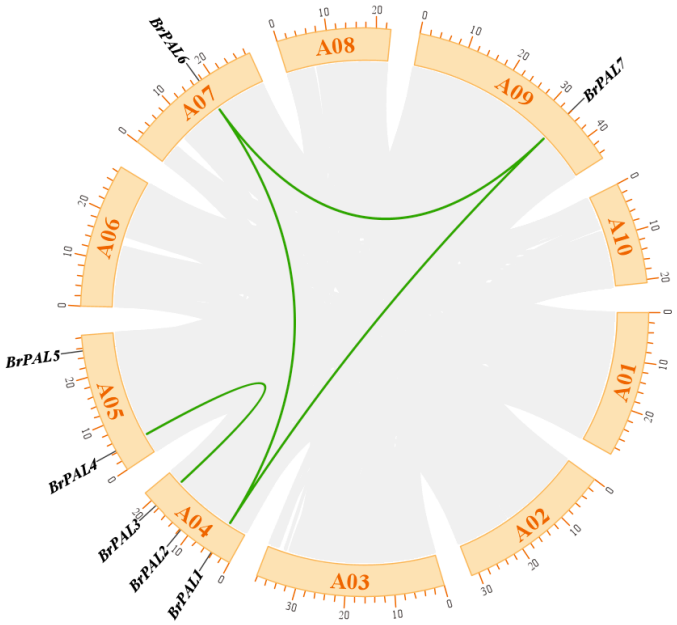

Figure 3. The distribution and segmental duplication of *PAL* genes in *Brassica rapa*, the yellow panel uses circles to show ten chromosomes A01-10 are *Brassica rapa* chromosome numbers, the green lines represent collinear gene pairs, and the gray areas are collinear regions.

To further infer the phylogenetic mechanism of the cabbage *PAL* family, the collinear relationship between *Brassica rapa* and *Arabidopsis* and *Brassica* crops (*Brassica napus* and *Brassica oleracea*) was explored. The results showed strong collinear regions between *Brassica rapa* and *Arabidopsis* (Figure 4a), *Brassica napus*, and *Brassica oleracea (*Figure 4b). *Brassica napus* (3262) had the most collinear regions with *Brassica rapa*, followed by *Brassica napus* (1728) and *Arabidopsis* (867), which is related to the genetic background of *Brassica napus* hybridization and chromosome doubling from *Brassica rapa* and *Brassica oleracea*. In addition, a homologous comparison analysis of the *PAL* gene pairs between *Brassica rapa*, *Arabidopsis thaliana*, *Brassica napus*, and *Brassica oleracea* was conducted, and the homologous gene pairs among the three were 10(At), 22(Bn), 26(Bo). Among them, *BrPAL1*, *BrPAL3*, *BrPAL4*, and *BrPAL7* had 2–5 homologous genes among all three species, *BrPAL2* and *BrPAL6* were not found homologous in *Brassica rapa* and *Arabidopsis*, and *BrPAL6* was not found homologous among all three species, probably due to the fact that *BrPAL6* originated after the divergence of these species. In addition to whole genome duplication events, these results show that independent duplication events occurred during the evolution of these species.

3.4. Structure and Conserved Motif Analysis of the Cabbage PAL Gene

Gene structure diversity is an important part of gene family evolution. To better understand the relationship between the phylogenetic evolution of *BrPAL* family members, gene structure, and conserved motifs, the exons/introns of conserved motifs were analyzed

for the structure and distribution and phylogenetic tree of the *BrPAL* family. The results are shown in Figure 5a,b, that *BrPAL* members with similar exon/intron numbers and positions clustered in the same branch of the phylogenetic tree. The *BrPAL2* gene has no UTR, the *BrPAL6* gene has no intron, the *BrPAL2* and *BrPAL5* genes contain one intron each, and the *BrPAL1*, *BrPAL3*, *BrPAL4*, and *BrPAL7* genes contain one intron each.

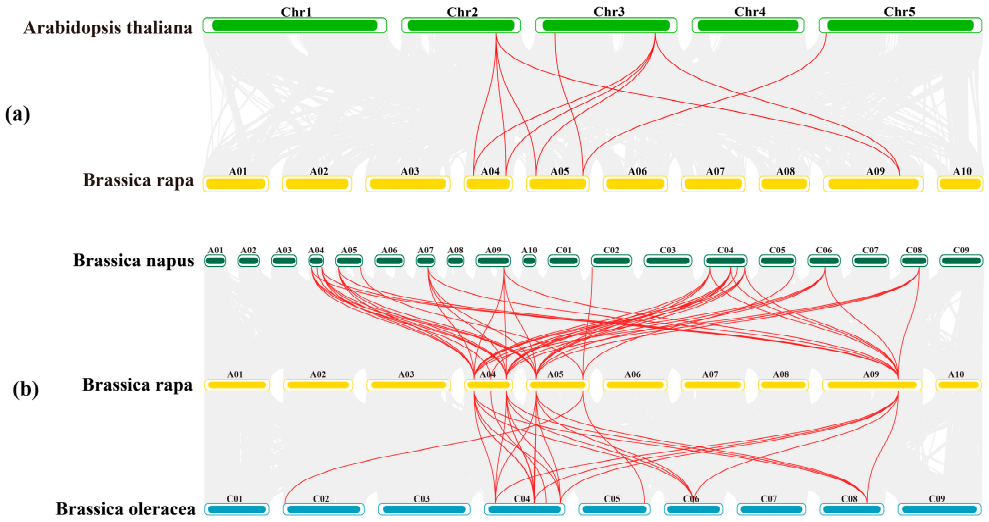

Figure 4. (**a**) Collinearity analysis between *Brassica rapa* and *Arabidopsis*, the gray line in the background indicates the collinear block in the genomes of *Brassica rapa* and *Arabidopsis*, and the red line highlight the homologous *PAL* gene pair; (**b**) *Brassica rapa*, *Brassica napu*, *Brassica oleracea* Collinearity analysis of rapeseed, the gray line in the background indicates the collinearity blocks in the genomes of *Brassica rapa*, *Brassica napus* and *Brassica oleracea*, and the red line highlights the homologous *PAL* gene pairs.

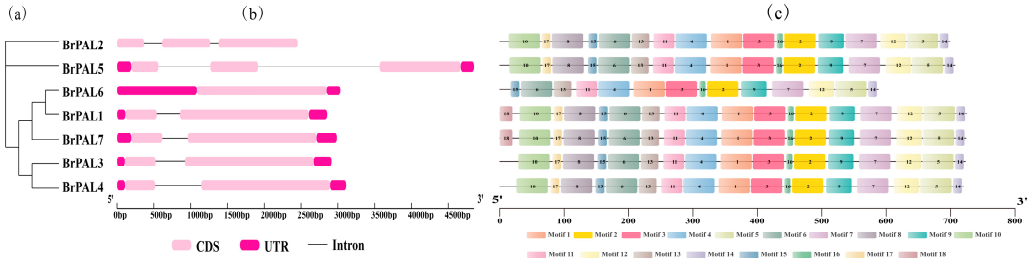

Figure 5. Evolutionary relationship, conserved protein motifs and gene structure of *BrPAL* gene. (**a**) The phylogenetic tree of the *PAL* gene in Chinese cabbage. The phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA 7.0, and the bootstrap was repeated 1000 times; (**b**) The exon-intron structure of the *PAL* gene in Chinese cabbage. Red represents UTRs, pink represents CDS, and thin black lines represent introns. (**c**) The motifs in the *BrPAL* protein were identified by the MEME program, and numbers 1–18 composed of motifs are displayed in boxes of different colors.

Proteins with highly consistent amino acid sequences, especially in the functional domains, often have similar biological functions. Using MEME online software to analyze the conserved motifs of cabbage *PAL* proteins (Figure 5c), the results show that each *BrPAL* contains 14 to 18 motifs in the length range of 11 to 50 amino acids, and the distribution is very similar. The three members' makeups differ in several ways. The process is responsible for different functions.

3.5. Analysis of Cis-Acting Elements in Promoters of the PAL Gene Family in Chinese Cabbage

The cis-acting elements in the promoter region are specific motifs that can combine with transcription factors to further participate in the expression and regulation of genes.

To understand the functions of potential cis-acting elements in the members of the *BrPAL* gene family, the promoter region of each member ATG upstream of a 2000bp sequence was extracted and used PlantCARE software was used to predict the cis-acting element (CRE) in the promoter region, and the common TATA box, CAAT box, and undefined cis-elements were used to select some CREs with more enrichment. Figure 6a,b show the enrichment and location of the cis-acting elements of the *BrPAL* gene family promoter, respectively. The results show that the *BrPAL* gene contains a large number of cis-acting elements, which can be mainly divided into responses to abiotic stress (104), light (103), growth and development (51), and plant hormones (102). Each *BrPAL* gene contains more than three elements related to abiotic stress, six of which are more abundant, including MYB, MYC, low-temperature response element (LTR), and stress response element (STRE); nine elements are related to light response, including G-box, box-4, and I-box; most *BrPALs* contain these light-responsive elements, indicating that the expression of *BrPAL* genes may be induced or inhibited by light; five elements are related to growth and development, including ARE, MSA-like, O2-site, GCN4-motif, and CCAAT-box; there are nine types including ABA response element (ABRE), salicylic acid response element (TCA-element), gibberellin response (P-box), MeJA response element (TGACGmotif and CGTCAmotif), auxin response elements (AuxRR-core and TGA-element) and other components associated with plant hormone response. The above results indicate that the accumulation of anthocyanins in purple cabbage may be related to various transcriptional regulation mechanisms of *BrPAL* family genes through abiotic stress, light, and different hormone-related cis-elements during plant growth.

Figure 6. Prediction of cis-acting elements in the *BrPAL* promoter. (**a**) Each *BrPAL* promoter contains the number of detected cis-acting elements, classified into four types; (**b**) TBtools visualizes the hormone response elements in the *BrPAL* promoter, including the position of the element and the type.

3.6. Expression Patterns of Cabbage PAL Genes under Different Light Quality, Abiotic Stress and Hormone Induction

To further explore the potential role of cabbage *PAL* genes under different light quality, abiotic stresses and hormone treatments, we analyzed their expression levels using qRT-PCR. As shown in Figure 7, all members were down-regulated under FR and FU treatment, and all genes were up-regulated under UVA treatment except for *BrPAL2* and *BrPAL5*. As shown in Figure 8, only *BrPAL2* and *BrPAL5* expressions were up-regulated under heat stress reaching peaks at 6 h and 3 h, respectively, while the rest of the genes remained at low expression levels; except for the downregulation of *BrPAL5*, the expression levels of other members were relatively high under treatment at low temperatures. The expression levels of *BrPAL1*, *BrPAL2*, and *BrPAL3* at 0–12 h and *BrPAL4* and *BrPAL6* at 0–24 h gradually increased with time, and the expression of *BrPAL6* increased in all members at 12 h of cold stress. Interestingly, the expression pattern of *BrPAL2* was consistent under high-temperature and low-temperature treatment; the expression of *BrPAL* gene family members was downregulated under PEG and NaCl treatment, but there was a tendency to recover at 12 h and 24 h. As shown in Figure 9, the expression of the *BrPAL* gene family showed a trend of significant downregulation under IAA treatment. The expression of *BrPAL1*, *BrPAL3*, *BrPAL4*, *BrPAL6*, and *BrPAL7* was the lowest at 3 h, and the expression of *BrPAL2* and *BrPAL5* reached the lowest at 12 h and gradually recovered at 24 h. Under ABA treatment, the expression levels of *BrPAL1*, *BrPAL3*, and *BrPAL4* decreased within 3–12 h; *BrPAL5*, *BrPAL6*, and *BrPAL7* gradually decreased within 3–6 h; and the expression level of *BrPAL2* reached the lowest level. The expression levels of all *BrPAL* genes were restored after 24 h of ABA treatment; under the treatment of MeJA, the expression levels of most genes in the *BrPAL* family were significantly upregulated, and the highest expression levels appeared at 3, 6, 12, and 24 h. The highest expression level of *BrPAL6* was 1.99 times higher than that at 0 h at 12 h, and only the expression levels of *BrPAL2* and *BrPAL3* decreased; under GA treatment, the expression of *BrPAL2* reached the lowest value at 12 h, and the expression levels of the rest of the family genes gradually decreased within 3–6 h and recovered from 12 to 24 h.

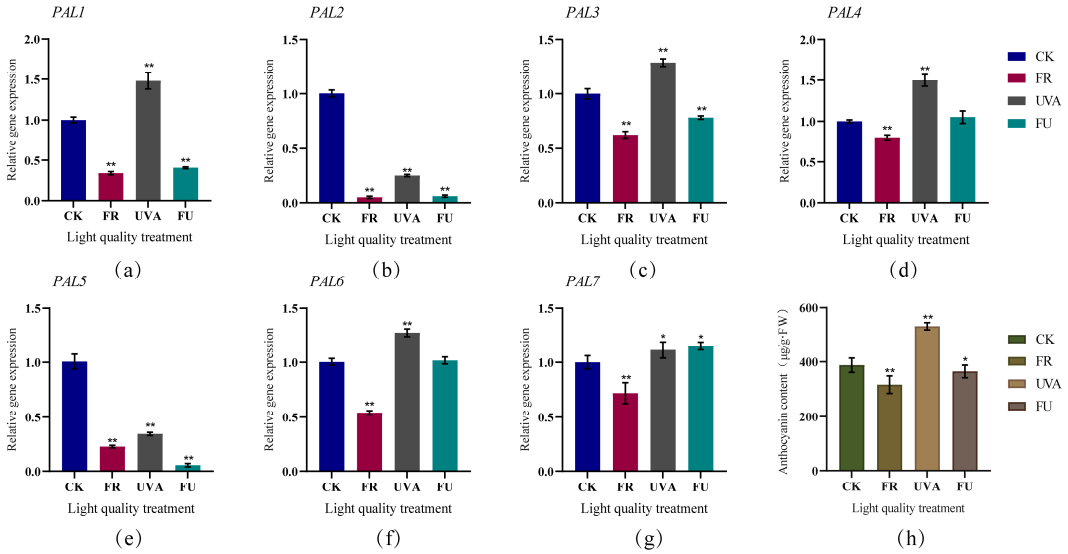

Figure 7. The relative expression level and anthocyanin content of *BrPAL* gene under light quality treatment. (**a**–**g**) Histogram of relative expression of *PAL* gene in purple cabbage under light quality treatment; (**h**) Histogram of anthocyanin content in purple cabbage under light quality treatment. Data represent the mean of triplicate with three biological replicates. * indicates significance ($p < 0.05$); ** indicates extremely significant difference ($p < 0.01$).

At the same time, the anthocyanin content of purple cabbage was measured under light-quality treatments, abiotic stress treatments, and hormone treatments. The results

(Figures 7h, 8h and 9h) showed that the anthocyanin content decreased under FR and FU treatment, and the anthocyanin content increased under UVA treatment; however, the amount of anthocyanin in the FU treatment was slightly higher than that of FR and close to that of CK. Under cold stress and MeJA treatment, the anthocyanin content gradually increased over 0–24 h; under the high temperature, drought, salt stress, IAA, ABA, and GA treatments, the cyanin content gradually decreased during over 0–24 h, and the anthocyanin content began to rise after 24 h under the IAA, ABA, and GA treatments.

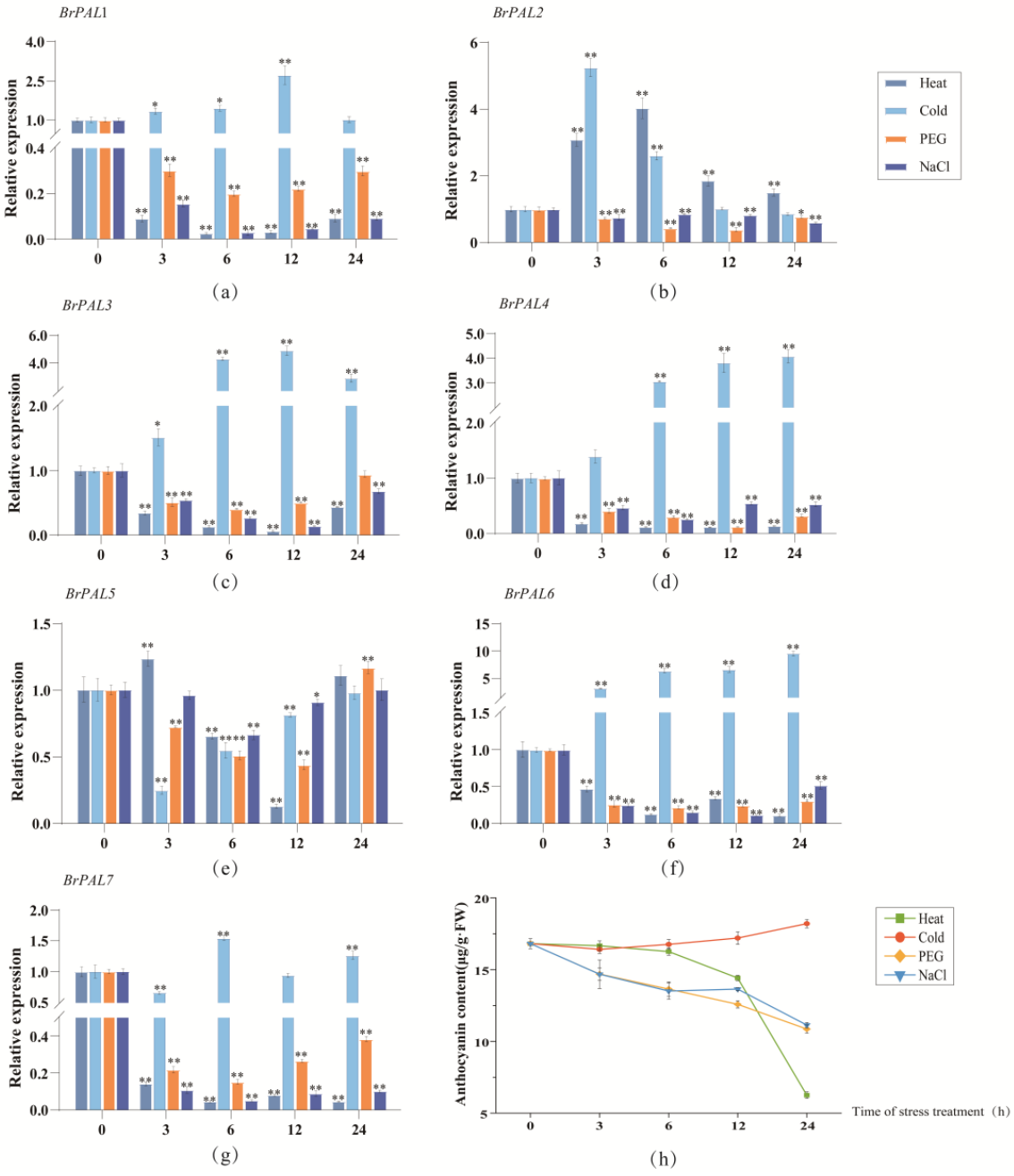

Figure 8. Relative expression level and anthocyanin content of *BrPAL* gene at 0, 3, 6, 12, 24 h under abiotic stress. (**a**–**g**) Histogram of relative expression of *PAL* gene in purple cabbage under high temperature, low temperature, drought, and salt stress; (**h**) line graph of anthocyanin content in purple cabbage under abiotic stress; Data represent the mean of triplicate with three biological replicates. * indicates significant difference (p value < 0.05); ** indicates extremely significant difference (p value < 0.01).

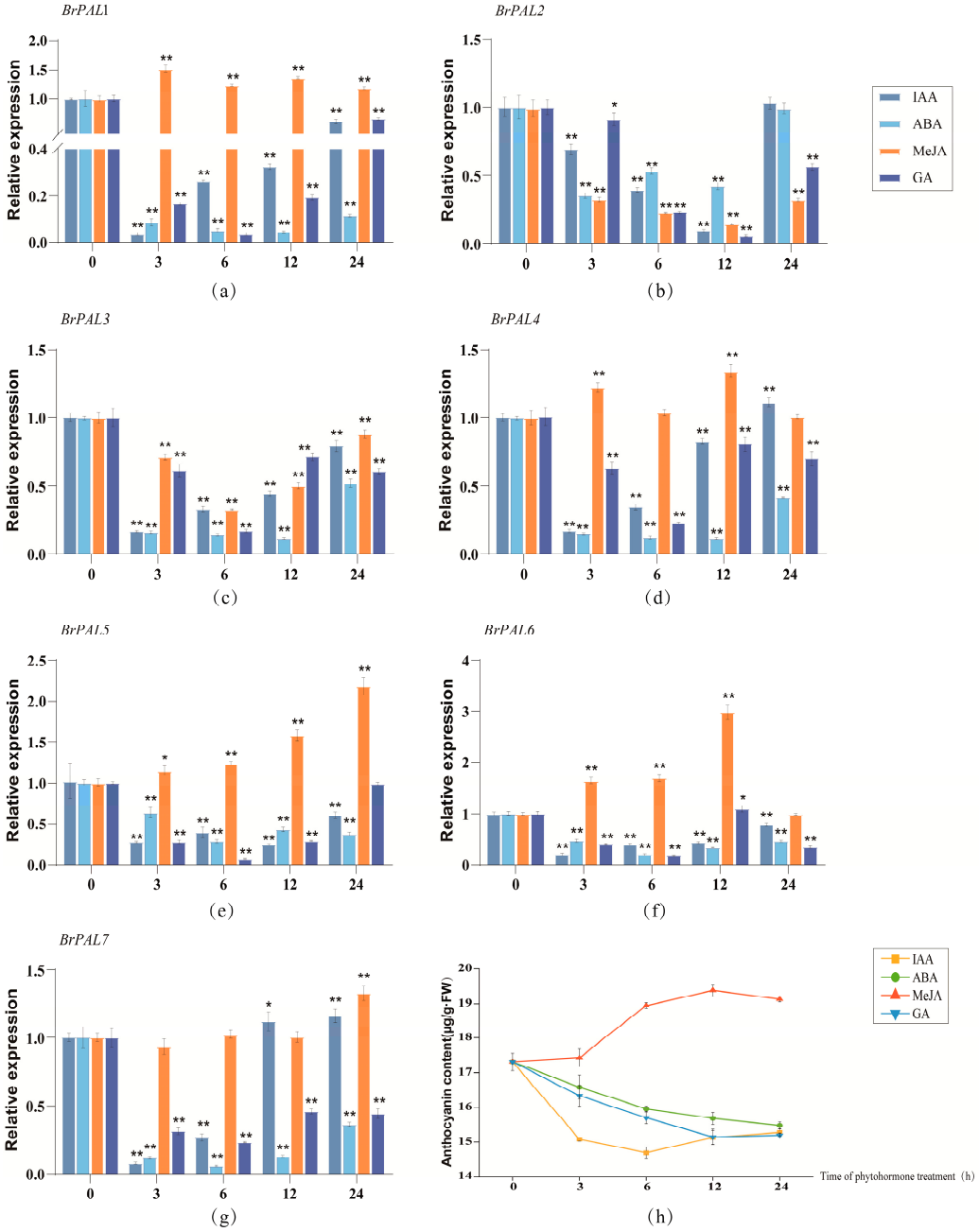

Figure 9. Relative expression level and anthocyanin content of *BrPAL* gene at 0, 3, 6, 12, 24 h under hormone treatment. (**a**–**g**) Histogram of relative expression of *PAL* gene in purple cabbage under IAA, ABA, MeJA, and GA; (**h**) line graph of anthocyanin content in purple cabbage under hormone treatment; Data represent the mean of triplicate with three biological replicates. * indicates significant difference (p value < 0.05); ** indicates extremely significant difference (p value < 0.01).

4. Discussion

Due to the key role of *PAL* in secondary phenylpropanoid metabolism [31], such as mechanical support (lignin), protection against biotic and abiotic stresses (antioxidants), pigments, such as anthocyanins, and flavonoid nodule factors [32], signal transduction has been extensively studied. At present, the function of the *PAL* gene has been discovered in many species. Amplification of *GmPAL1.1* in *Arabidopsis* promoted seed viability at physiological adulthood under high temperature and high humidity (HTH) stress [33], poplar leaf injury stimulated the upregulation of *PtPAL1* expression [34], the *TaPAL* family was important in the defense against wheat stripe rot [17], pepper plants silenced by *CaPAL1*

expressed Xcv susceptibility [34], *AtPAL1/AtPAL2/AtPAL3/AtPAL4* quadruple mutants showed dysplasia, and the accumulated salicylic acid level was also greatly reduced [14]; Nonetheless, the role of the *PAL* gene family in Chinese cabbage has not been identified and studied systematically.

In this research, based on the cabbage genome, we identified seven *BrPAL* genes with the same *PAL* gene family members in the tea tree [11], hickory nut [5], and cucumber [7]. With similar gene structures and conserved motifs, the *BrPAL* family in the same lineage has an extremely conserved gene structure and motif set, and these conserved motifs may be the basic elements that determine the common molecular functions of the *PAL* family in different plant species [34]. Phylogenetic analysis of Chinese cabbage, *Brassicaceae*, *Arabidopsis*, and *Brassica* crops showed that the same subgroup contained the *PAL* genes of Chinese cabbage and *Arabidopsis*. *BrPAL* exhibits a close relationship with two other *Brassica* crops (*Brassica napus* and *Brassica oleracea*) and *Arabidopsis*. Four *BrPAL* genes were homologous to *Arabidopsis* and *Brassica* crops, but the number of *BrPAL*, *BnPAL*, and *BoPAL* genes were significantly increased compared with *Arabidopsis PAL* genes, probably because they have undergone genome-wide triploidization events in *Brassica* since they diverged from the *Arabidopsis* lineage, indicating a high genetic diversity of *TaPAL* [35,36]. In Chinese cabbage, *BrPAL* family members are not distributed on all chromosomes but rather are distributed unevenly on four chromosomes, which is similar to the situation in which *PAL* genes are only distributed on a few chromosomes in many species [34]. During plant evolution, Whetten and Sederoff discovered that the reproduction and change in ancestors have the ability to generate several family members, which may be aggregated on a single chromosome or spread across multiple chromosomes [17,29], demonstrating that genome and chromosomal duplication are driving the expansion of the *BrPAL* family, While variations in the size of gene families could account for the wide range of appearances among *Brassica* species [37]. In addition, functional redundancy among closely related family members can be estimated and can stimulate more effective strategies for identifying defective phenotypes in loss-of-function studies in cabbage breeding.

The *PAL* gene's promoter activity also controls the gene's growth and induction of expression, and the promoter region usually contains a variety of cis-regulatory elements [9]. The results of the prediction and analysis of the *BrPAL* promoter's cis-acting elements indicate that *BrPAL* may respond to light, abiotic stress, and different hormones. Based on the UVA, FR, high-temperature, low-temperature, drought, salt stress, IAA, ABA, and MeJA treatments of PAL family genes, qRT-PCR expression analysis of purple leaf cabbage seedlings showed that under light quality treatment, most members have the same expression pattern. Under UVA treatment, FR is upregulated, and FU treatment is downregulated. Only *BrPAL2* and *BrPAL5* were downregulated, and the G-box in the promoters of *BrPAL2* and *BrPAL5* was more abundant than that in other members. This may be the reason for the different expression patterns of *BrPAL2* and *BrPAL5* from those of other members. Under low-temperature conditions, the expression of all members, except *BrPAL5*, was up-regulated, and under high-temperature conditions, the expression of most members was down-regulated, with the exception of *BrPAL5* and *BrPAL2*. This suggests that the differential regulation of *BrPAL* expression may be due to the different cis-elements present in each member. Compared with other members, *BrPAL5* has a less obvious response to abiotic stress, which may be related to the presence of fewer abiotic stress response elements in the promoter sequence; the jasmonate response element in *BrPAL6* is the most abundant, and its expression is upregulated. This most notably shows that the expression of the *BrPAL* gene is in good agreement with its promoter prediction.

Studies on other species also found similar results to this study. The expression levels of turnip *PAL* [38] and tomato *SlPAL5* [39] were upregulated under UVA irradiation. Under UVA irradiation, the expression levels of the majority of *BrPAL* were likewise increased in this investigation. The expression levels of the *CsPAL* genes were all upregulated under cold stress [7], which is consistent with the upregulation of the expression levels of the six *BrPALs* at low temperatures in this study. The *AtPAL1/AtPAL2* double mutants in

Arabidopsis showed a higher drought tolerance [14], which is consistent with the decreased expression of *BrPAL* under drought stress in this study, indicating that *BrPAL* has a negative regulatory effect on drought stress. The expression of *CsPAL2* and *SlPAL5* decreased after the exogenous ABA treatment [40]. The expression of *BrPAL* was also downregulated under ABA treatment; six of the potato *PAL* genes were significantly upregulated, while four were significantly downregulated under MeJA treatment [10]. In this study, five *PAL* genes of cabbage were significantly upregulated, while two were significantly downregulated under MeJA treatment. In summary, purple cabbage can regulate the expression of most *PAL* genes under light quality, various abiotic stresses, and phytohormone treatments, with different expression patterns under different light qualities. Differences in the timing with stress and phytohormone treatment can differentially induce the expression of *BrPAL* gene family members, and such differences in gene expression are also advantageous and may allow plants to have different levels of stress resistance, as well as an important role in hormone signaling. In the meanwhile, the changes in anthocyanin content in purple cabbage under light quality, abiotic stress, and hormone treatments were mostly positively correlated with the changes in the expression level of the *BrPAL* gene, demonstrating a strong correlation between the *BrPAL* gene's level of expression and the anthocyanin content of purple cabbage.

5. Conclusions

In this study, we identified seven members of *BrPAL* located on four chromosomes that play key roles in the anthocyanin biosynthetic metabolic pathway. We systematically and comprehensively analyzed the *BrPAL* family, including phylogenetic analysis, conserved structural domains, chromosomal location, gene structure and gene expression. Expression of *BrPAL* genes under light quality, stress and hormone treatment showed a highly significant correlation with their promoter cis-elements and anthocyanin content in purple-leaf cabbage. Taken together, the results of this study provide new clues for the functional study of *BrPALs* and further construction of the anthocyanin regulatory network in cabbage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9040469/s1, Table S1 Primer sequences used for qRT-PCR amplification of seven *BrPAL* genes; Table S2. Ka/Ks analysis of *BrPAL* duplicated genes.

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