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Cloning and Expression Analysis of *PAP1* in *Brassica juncea*

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Abstract Anthocyanins are important substances accounting for the leaf color in *Brassica juncea* and *PAP1* gene is one of the key transport factors in the anthocyanin synthesis pathway. In this study, homologous cloning technology was used to clone the *PAP1* gene sequences of *Brassica juncea* with different leaf colors. Specific primers were designed according to the gene sequences of *Brassica rapa* with high homology for PCR amplification. The *PAP1* gene of *Brassica juncea* is 1 348 bp~1 669 bp long, and the coding region sequence is 744 bp~753 bp, including 3 exons and 2 introns. Two MYB blinding domains are found in PAP1 protein at the site of 9~59 and 62~110 amino acids. Phylogenetic analysis showed that the *PAP1* gene of *Brassica juncea* had high homology with the related genes of *Brassica rapa* and *Brassica rapa* subsp. rapa, but had low homology with *Arabidopsis thaliana*. Compared gene sequences in *Brassica juncea* with different leaf colors, there are no differences between the coding sequence of purple and red leaf *Brassica juncea*, but the encoded protein have 22 amino acid differences from green leaves. We also observed the lower expression level of *PAP1* and its related target genes such as *DFR*, *TT19* in green leaves, which may lead to the differences of leaf color in *Brassica juncea*. This study provides a reference for exploring the function of *PAP1* gene and the formation mechanism of different leaf color of *Brassica juncea*.
Keywords *PAP1* gene; Anthocyanin; Gene cloning; Expression analysis

Anthocyanin is a class of water-soluble natural pigment that exists widely in nature, and it is an important secondary metabolite in plants, which could make plant leaves and petals show colorful colors (Fu et al., 2018). The accumulation of anthocyanins could help plants enhance free radical scavenging and antioxidant, resist environmental stresses such as low temperature and drought, and protect the tissues for photosynthesis (Butelli et al., 2012; Kim et al., 2017). Meanwhile, edible foods rich in anthocyanins have many biological health care functions, such as fighting cancer and improving cardiovascular (Puiggròs et al., 2014). Therefore, plant resources rich in anthocyanins are highly valued by breeders, and have great application prospects in the breeding of ornamental, vegetable and stress resistant varieties.

The transcription of the key enzyme genes for anthocyanin synthesis is mainly regulated by the MBW complex, including MYB, bHLH and WD40 transcription factors. Among them, MYB transcription factor is the most numerous, which plays an important role in regulating the synthesis and accumulation of anthocyanins (Dubos et al., 2010; Xu et al., 2014; Yao et al., 2017). The proteins encoded by the *PAP1* (*Production of Anthocyanin Pigment 1*) gene belongs to R2R3 MYB transcription factor, which can regulate the expressions of *DFR*, *ANS/LDOX*, *TT19*, *TT8*, *GL3*, *EGL3* and other genes to promote the synthesis and accumulation of anthocyanins (Maier et al., 2013; Yan et al., 2019). In *Brassica oleracea* (*CC*, 2n=18), the up-regulated expression of *BoPAP1* is an important reason for the appearance of purple leaf traits. However, it is not completely clear whether there is a similar mechanism in other *Brassica* species.

Brassica juncea (AABB, 2n=36) belongs to *Brassica* of Brassicaceae, which is an important raw material of oil and vegetable crop in China, with abundant variation resources (Yang et al., 2016; Yang et al., 2018). Some genes related to the color of the seed coat of *Brassica juncea* have been cloned, while there are fewer studies on cloning and expression of gene related to leaf anthocyanin synthesis (Yan et al., 2007; Yan et al., 2011). *Brassica rapa*

(AA, 2n=20), as the ancestor of *Brassica juncea*, provides a good sequence reference for homologous cloning because its anthocyanin synthesis pathway is relatively clear (Guo et al., 2014).

The purpose of this study is to clone the *PAP1* gene from *Brassica juncea* with different leaf colors through the method of homologous cloning. And we also want to use bioinformatics methods to predict the structure and function of the protein encoded by the *PAP1* gene, to compare its sequence and expression differences among leaves with different colors, and to explore the relationship between *PAP1* gene and leaf color. This study provides a reference for exploring the function of *PAP1* gene and the formation mechanism of different leaf color of *Brassica juncea*.

1 Results and Analysis

1.1 The phenotype of *Brassica juncea* with different leaf colors

The whole leaves of *B. juncea* (*lv*) are green. *B. juncea* (*hong*) is lavender overall and the purple radiates from the edge of the leaf to the middle, while the veins are green. The leaves and veins of *B. juncea* (*zi*) are dark purple. Their phenotypes are obviously different (Figure 1A). Moreover, it was found that the anthocyanin content from high to low is *B. juncea* (*zi*), *B. juncea* (*hong*) and *B. juncea* (*lv*) by measuring. The anthocyanin content in *B. juncea* (*zi*) is twice that of *B. juncea* (*hong*) and 9 times that of *B. juncea* (*lv*). Their anthocyanin content is also obviously different (p<0.01) (Figure 1B). Therefore, we could know the phenotype of *Brassica juncea* with different leaf colors related to anthocyanin content.

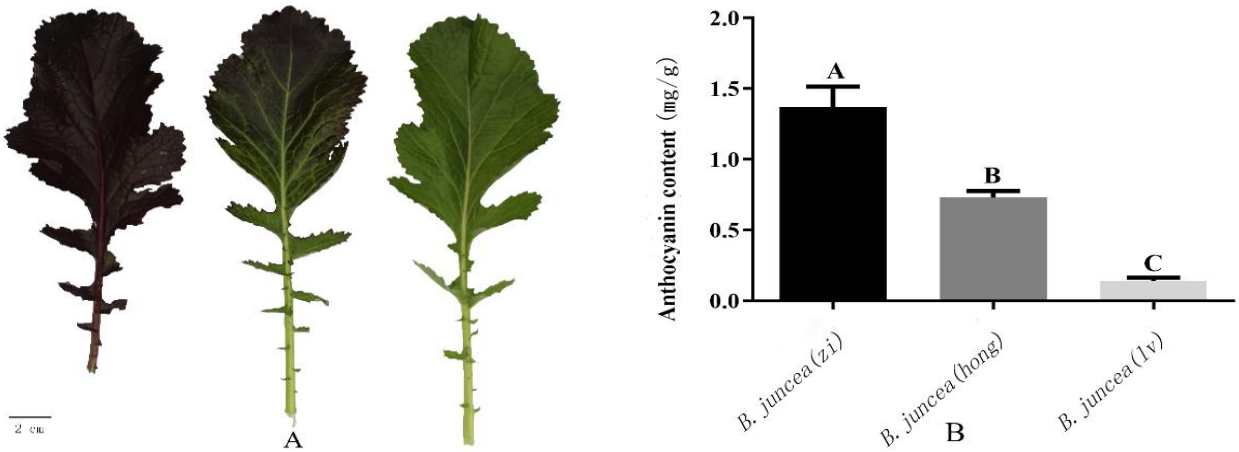

Figure 1 Comparison of leaf phenotype and anthocyanin content of *Brassica juncea* with different leaf colors

Note: A: The leaf types from left to right are *Brassica juncea* of purple, red and green; B: Different capital letters indicate significant differences at 0.01 level

1.2 Cloning of *PAP1* gene in *Brassica juncea*

Brassica rapa and *Brassica juncea* are both belongs to *Brassica*, and *Brassica rapa* is the ancestor of *Brassica juncea*, so they two have high homology. We used the DNA of *Brassica juncea* of purple, red and green as templates and designed homologous primers for PCR amplification by using the *BraPAP1* gene sequence of *Brassica rapa*. After agarose gel electrophoresis, we could see base pair is like a single strip with higher brightness, the number of which is 1 546, 1 669, 1 664, 1 348, 1 598 (Figure 2). Then five *PAP1* gene sequences in *Brassica juncea* with different leaf colors were successfully cloned.

1.3 Sequence analysis of *PAP1* gene in *Brassica juncea*

Through homologous cloning, the 5 sequences with higher homology were labeled as *B.juncea* (*zi*), *B.juncea* (*zi-2*), *B.juncea* (*lv*), and *B.juncea* (*lv-2*), *B.juncea* (*hong*). The GenBank accession numbers of the cloned *PAP1* genes are MT210230, MT210231, MT210232, MT210233 and MT210234. The sequencing results showed that the *PAP1* gene length of *Brassica juncea* is from 1 348 bp to 1 669 bp, and the CDS length is from 744 bp to 753 bp. It also showed the gene sequence includes 3 exons and 2 introns, and the number of encoded amino acids is from 247 to 250 (Table 1).

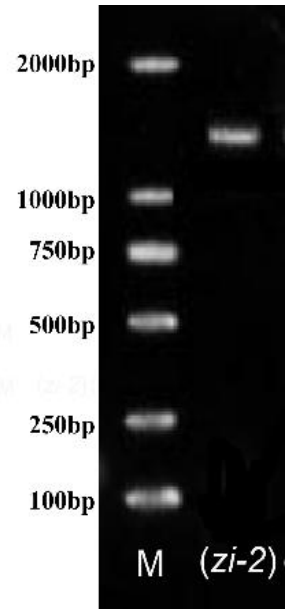

Figure 2 Amplification results of primer *PAP1.2* in purple leaf
Note: M: DL2501 DNA marker; (zi-2): PCR product of purple leaf

Table 1 Basic information of *PAP1* gene in different leaf colors *Brassica juncea*

Name	Gene length (bp)	CDS length(bp)	Number of encoded amino acids	*B.rapa* homologous gene number	E value
B. juncea(zi-2)	1 546	753	250	Bra001 917	0
B. juncea(zi)	1 669	744	247	Bra004 162	0
B. juncea(lv-2)	1 598	750	249	Bra039 763	0
B. juncea(lv)	1 348	744	247	Bra004 162	0
B.juncea(hong)	1 664	744	247	Bra004 162	0

1.4 Bioinformatic analysis of *PAP1* gene

Bioinformatic analysis showed that the theoretical molecular weight of the protein encoded by the *PAP1* gene is from 27 941.7 to 28 613.4 and the theoretical isoelectric point is from 8.75 to 9.17, which proved that the protein is a basic protein. Using the online website ProtParam to analyze the proportion of amino acids in the PAP1 protein, we got the result that Leucine (Leu) has the highest amino acid content, which accounts for more than 10%. Using the ProtScale website (https://web.expasy.org/cgi-bin/protscale/protscale.pl/) to analyze the hydrophilicity and hydrophobicity of the PAP1 protein, we found that PAP1 has no typical hydrophobic region, which showed PAP1 protein is hydrophilic.

The secondary structure of *PAP1* gene is mainly composed of α-helix (32.79%~38.96%), extended strand (9.31%~12.96%), β turn (8.91%~12.80%) and random coil (38.80%~45.34%) (Table 2). Using SWISS-MODEL (https://swissmodel.expasy.org/) software, we constructed tertiary structure of PAP1 protein (Figure 3).

Table 2 Physicochemical properties of PAP1 protein in *Brassica juncea* with different leaf colors

Name	Protein molecular weight (d)	pI	α-helix (%)	Extended strand (%)	β turn (%)	Random coil (%)	Highest content amino acids	Lowest content amino acids
B. juncea(zi-2)	28 521.7	8.95	37.20	11.20	12.80	38.80	Leu	Gln
B. juncea(zi)	27 941.7	8.75	35.63	9.31	11.34	43.72	Leu	Phe, Tyr
B. juncea(lv-2)	28 613.4	8.40	38.96	10.84	9.24	40.96	Leu	Met
B. juncea(lv)	28 036.9	9.17	32.79	12.96	8.91	45.34	Leu	Tyr
B.juncea(hong)	27 941.7	8.75	35.63	9.31	11.34	43.72	Leu	Phe, Tyr

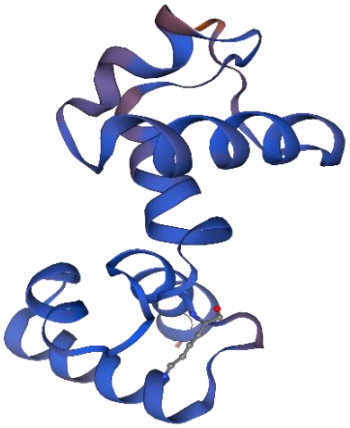

Figure 3 Prediction of tertiary structure of PAP1 protein in *Brassica juncea*

Using the SMART website (http://smart.embl-heidelberg.de/) to predict and analyze the conserved domain of PAP1 protein and using the online website Plant-mPLoc to predict and analyze the subcellular location of PAP1 protein, we got the result that the conserved domain of PAP1 protein includes two typical SANT binding domains (MYB binding domains), which respectively locates from the 9th to 59th and from 62nd to 110th of amino acid sequence (Figure 4). Subcellular location predicted that the protein is located in the nucleus. It was further proved that the cloned gene is the *PAP1* gene of R2R3 MYB transcription factor family.

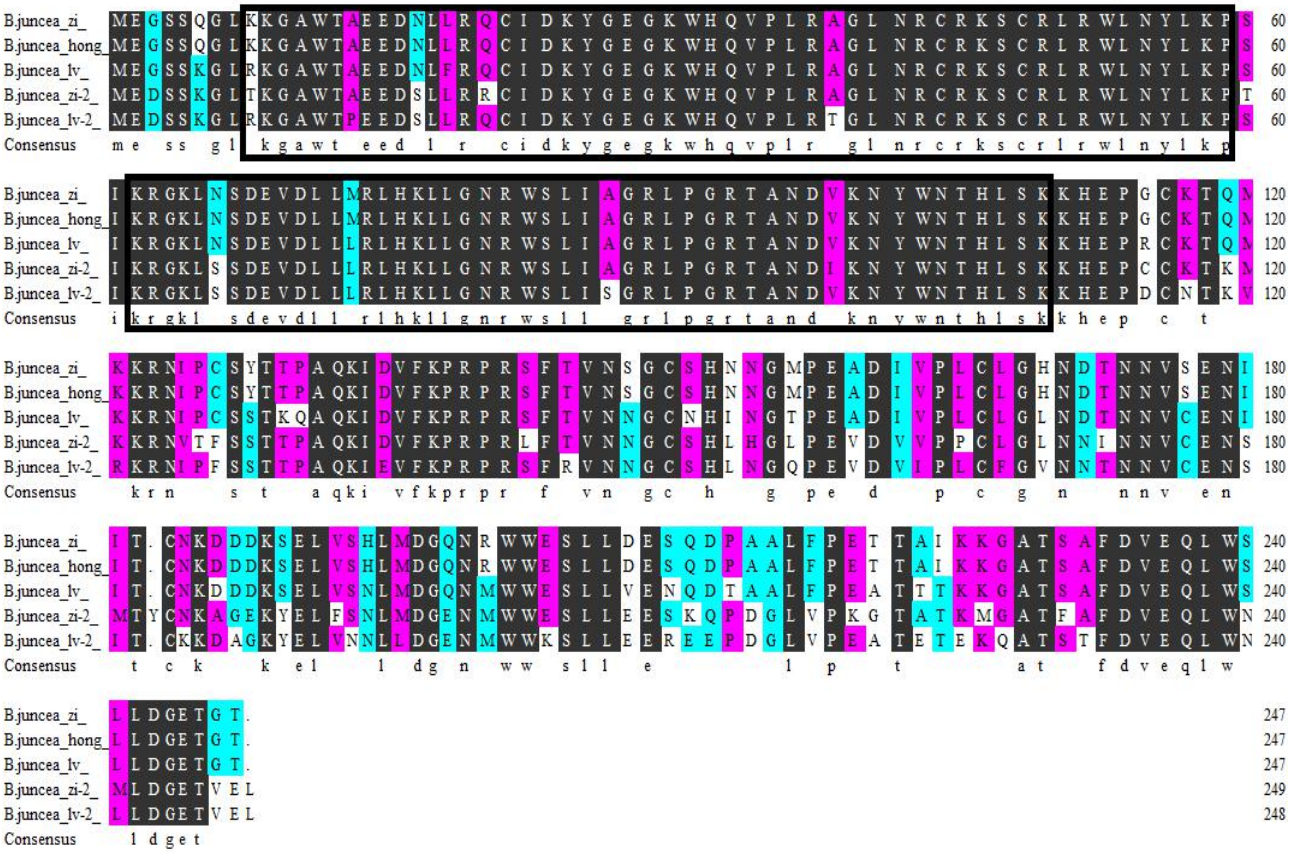

Figure 4 Comparison of amino acid sequences encoded by *PAP1* gene in *Brassica juncea* with different leaf colors

Note: The background color black indicates the same amino acid, and there is a difference in the amino acid sequence of the unlabeled black; the black box indicates the conserved SANT domain

1.5 Phylogenetic analysis of the coding sequence of *PAP1* gene

Putting the PAP1 protein sequence in *Brassica juncea* into the NCBI database for Blast search and comparison, and selecting the amino acid sequence of the relative plant with the highest homology to PAP1 for multiple sequence alignment and analysis, and then using MEGA v.7 software to build a phylogenetic tree, we got the result that the amino acid sequence of PAP1 in *Brassica juncea* has the highest homology with the amino acid sequence of *B.rapa*. The homology is from 92% to 100%. Among them, *B.juncea* (*zi*), *B.juncea* (*lv*) and *B.juncea* (*hong*) have high homology with *Bra004162* in the Brassica database. While *B.juncea (zi-2*) and *B.juncea (lv-2)* have high homology with *Bra001917* and *Bra039763*, respectively. It showed that the PAP1 protein is highly conservative in evolution. The amino acid sequence of PAP1 in *Brassica junce*a also has high homology with other relative plants of the same genus such as *B.napus*, *B.rapa subsp. rapa*, *B.oleracea var. botrytis* and so on. The homology is from 89% to 98%. The phylogenetic tree showed that the model plant *A.thaliana* is distributed outside the phylogenetic tree, indicating that the PAP1 protein in *A.thaliana* is relatively distant from the PAP1 protein in *Brassica juncea* (Figure 5).

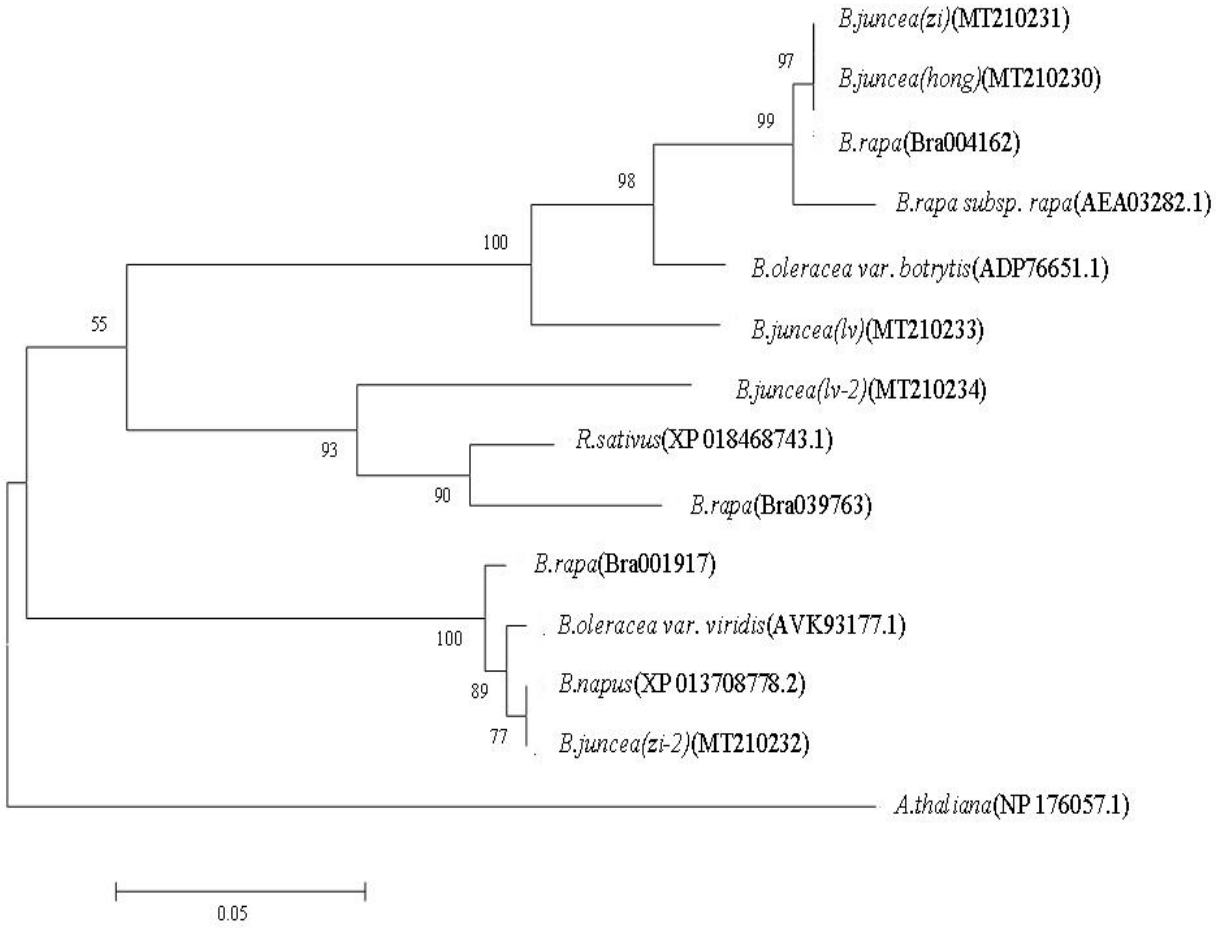

Figure 5 Phylogenetic analysis between PAP1 protein of *Brassica juncea* and other relative plants

1.6 Comparison of *PAP1* gene sequence in *Brassica junce*a with different leaf colors

It was found that there are certain differences among the *PAP1* gene sequence in *B.juncea*(*zi*), *B.juncea*(*lv*) and *B.juncea*(*hong*) by homology comparison analysis. The differences between *PAP1* gene sequence in *B.juncea*(*zi*) and that in *B.juncea*(*hong*), which are both homologous to *Bra004162*, are mainly concentrated in the intron region (Figure 6). But the coding sequence and amino acid sequence of the two are exactly the same (Figure 4). The sequence differences between *B.juncea*(*zi*)/*B.juncea*(*hong*) and *B.juncea*(*lv*) also exist in the coding region. Although *B.juncea*(*zi*)/*B.juncea*(*hong*) and *B.juncea*(*lv*) encode 247 amino acids, there are 22 differences in the amino acid sequence. Therefore, it was speculated that the difference of *PAP1* gene sequence in *Brassica juncea* may be the reason for difference of leaf color.

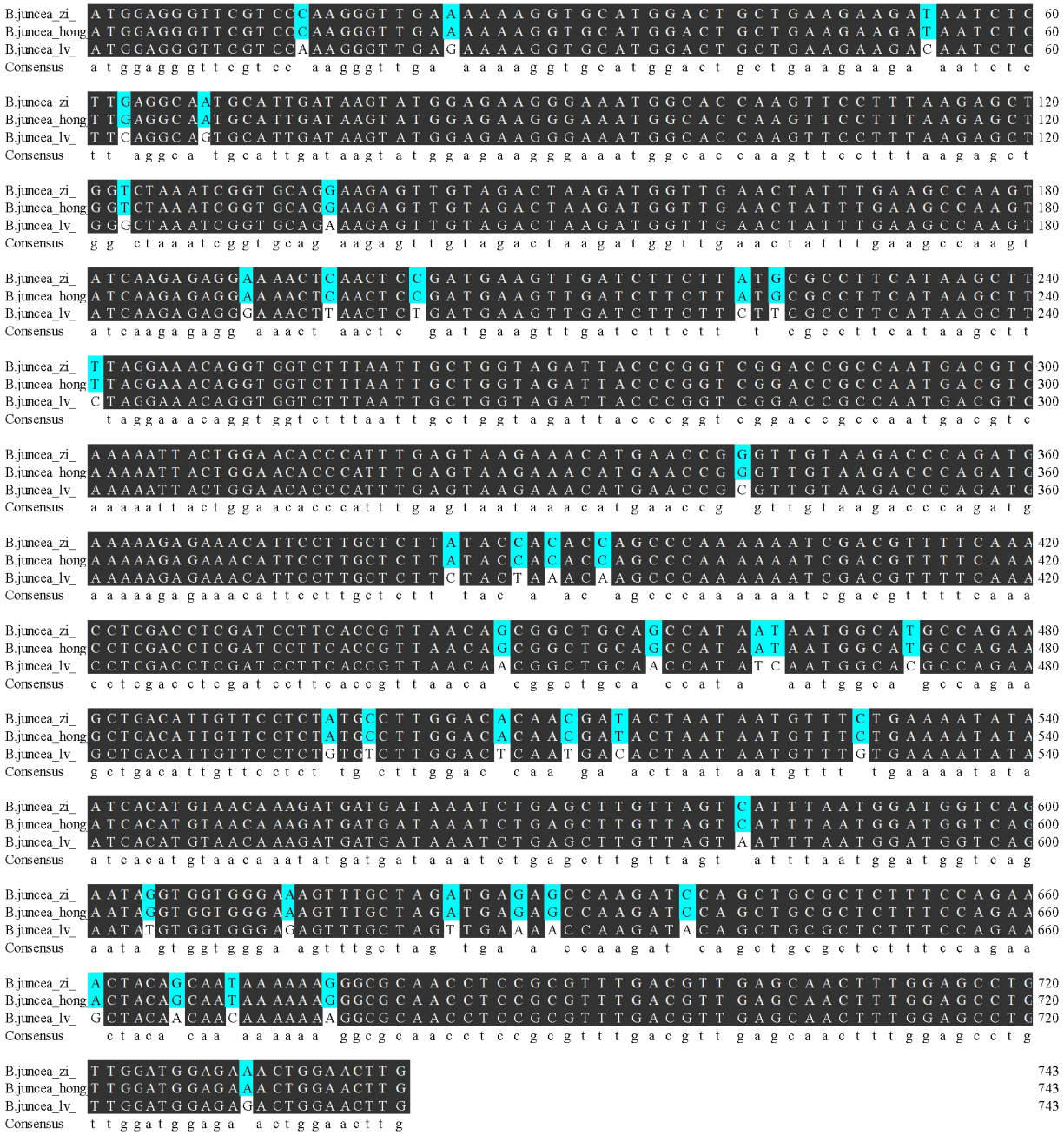

Figure 6 CDS sequences comparison of different leaf colors *Brassica juncea PAP1* gene that homologous to *Bra004162* of *B.rapa*

1.7 Expression analysis of *PAP1* gene and its regulatory genes

Extracting RNA from young leaves of *B.juncea* (*zi*) and *B.juncea* (*lv*) at the three-leaf stage, and using quantitative PCR to analyze the expressions of *PAP1* gene and its downstream genes *DFR*, *TT19*, *TT8* and so on, we got the result that the expressions of *PAP1* gene and its downstream genes *DFR*, *TT19*, *TT8*, *GL3* and *EGL* in *B.juncea* (*lv*) is lower than that in *B.juncea* (*zi*) (p<0.05), and the expressions of *DFR* and *TT19* in *B.juncea* (*zi*) is 5 to 7 times higher than that in *B.juncea* (*lv*) (Figure 7). It was known that the transcription factor encoded by the *PAP1* gene controls the expressions of downstream genes and affects the synthesis of anthocyanins. Furthermore, it was also known that the expression difference of each gene is correlated with the leaf color of *B.juncea* (*zi*) and *B.juncea* (*lv*). Therefore, it was speculated that different leaf colors may be caused by different expressions of genes.

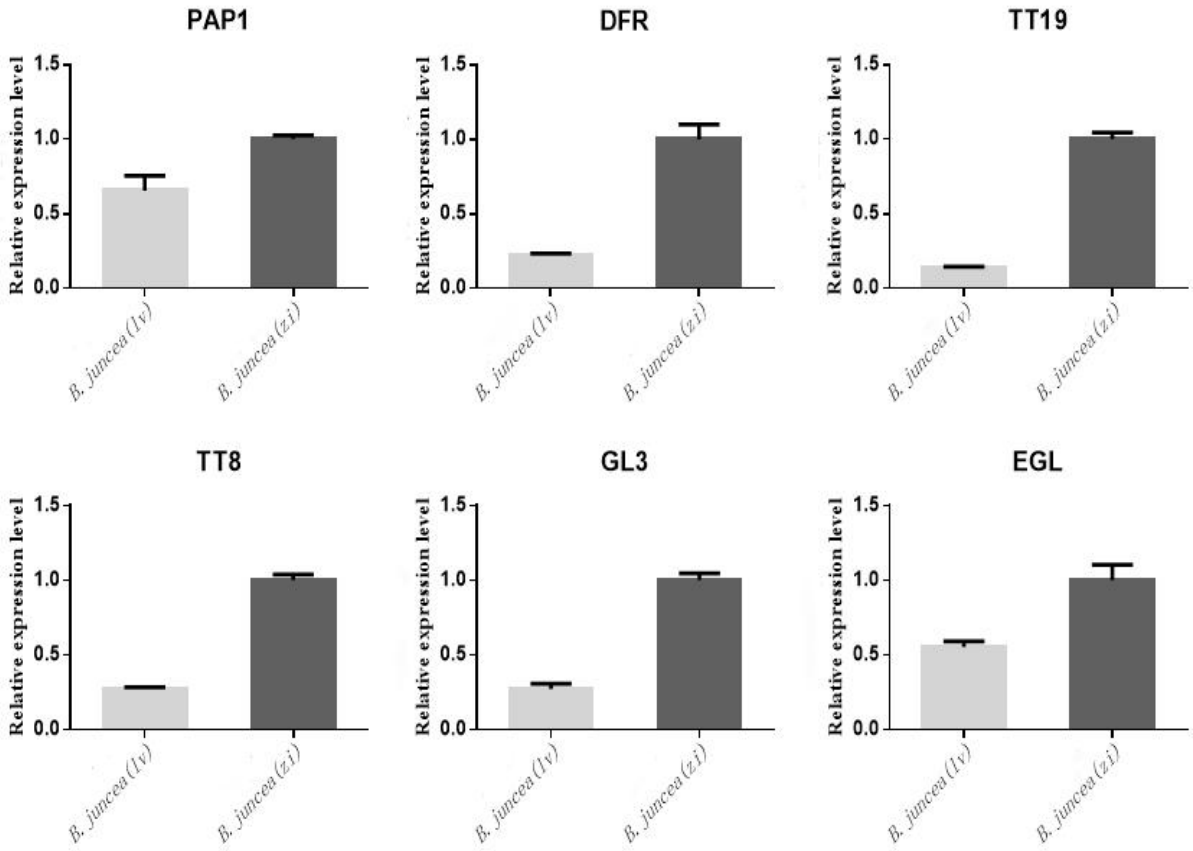

Figure 7 Comparison of the expression levels of *PAP1* and its downstream regulatory genes in *Brassica juncea* with different leaf colors

Through the cloning and expression analysis of the *PAP1* gene in *Brassica juncea* with different leaf colors, it was found that the evolution of the *PAP1* gene is extremely conservative. Moreover, there are different sequences and expressions of *PAP1* gene in *B.juncea* (*zi*) and *B.juncea* (*lv*). The expressions of *PAP1* and its downstream genes are significantly down-regulated in *B.juncea* (*lv*). In a word, it was speculated that the above differences may be the reason for appearance of different leaf colors of *Brassica juncea*.

2 Discussion

Anthocyanin is important secondary metabolites in plants. *Brassica juncea* is rich in genetic resources and crops with high anthocyanin content have great application prospects in ornamental, vegetable and stress resistance. Jeon et al. (2018) conducted a comprehensive analysis of the transcriptome and metabolome of *B. oleracea* (*lv*) and *B. oleracea* (*hong*). It was found that *B. oleracea* (*hong*) contains more anthocyanins than *B. oleracea* (*lv*) and the expression of anthocyanin synthesis gene is positively correlated with anthocyanin content. Moreover, it was found that the anthocyanin content in *B. juncea* (*zi*) is significantly higher than that in *B. juncea* (*hong*) and *B. juncea* (*lv*), and the expressions of related genes involved in the anthocyanin synthesis in *B. juncea* (*zi*) is higher than that in *B. juncea* (*lv*), indicating that anthocyanin is the main reason for leaf color changes.

The anthocyanin synthesis involves several structural genes and regulatory genes, among which, MYB is the most important and most numerous transcriptional regulatory factors. According to the number of its structural domains, it can be divided into four categories, namely 1R-MYB, R2R3-MYB, 3R -MYB and 4R-MYB. The expressions of R2R3 MYB transcription factors *PAP1* and *PAP2* can increase the expressions of structural genes in anthocyanin biosynthesis. In recent years, a large number of studies have shown that *PAP1* is a key transcription factor of MYB that regulates anthocyanin synthesis. The expression of anthocyanin was up regulated by binding to the promoter of the target gene, thus promoting the accumulation of anthocyanins (Borevitz et al., 2000). Liu et al.

(2017) cloned the *Arabidopsis thaliana AtPAP1* gene from the *Arabidopsis thaliana* inflorescence. They constructed a gene expression vector and transferred it into tobacco for heterologous gene expression. The results showed that the tobacco plants show different colors, indicating that the overexpression of *PAP1* gene has an effect on the color of tobacco plants. In this study, five *PAP1* gene sequences of *Brassica juncea* with different leaf colors were cloned by using homologous cloning technology. Through structural domain analysis, it was found that the *PAP1* gene in *Brassica juncea* contains two conserved domains, namely the MYB binding domain, which can be combined with the target gene promoter to induce the expressions of downstream genes, and its structure is consistent with the predicted function, which showed that this gene plays a role in anthocyanin biosynthesis. The homologous alignment of the gene sequence showed that the *PAP1* gene has high homology with the homologous sequence of *Brassica rapa*. Phylogenetic tree analysis showed that the PAP1 protein also has high homology with other related plants of the *Brassica* genus, and it is relatively distant from *A.thaliana*, which indicated that the gene is conservative in evolution. Compared with *A.thaliana*, *Brassica* experiences genome triploidization, so the *PAP1* gene also has multiple copies in *Brassica juncea* (Wang et al., 2011; Yang et al., 2016). In this study, five sequences were cloned, of which three were homologous to *Bra004162*, one was homologous to *Bra001917* and one was homologous to *Bra039763*. Among them, the amino acid sequence of PAP1 in *B.juncea* (*zi*) and *B.juncea* (*hong*) is exactly the same, but there are 22 differences from the amino acid sequence of *B.juncea* (*lv*). Gene expression analysis showed that *PAP1* gene and its downstream regulatory genes are down-regulated in *B.juncea* (*lv*), leading to lower anthocyanin content than *B.juncea* (*zi*). The up-regulated expression of *BoPAP1* in *Brassica oleracea* is an important reason for the appearance of purple leaf (Zhang et al., 2012). Therefore, we speculated that different sequences and expressions of *PAP1* gene may lead to the differences of leaf color in *Brassica juncea*. The specific mechanism of the appearance of color difference remains to be further studied.

3 Materials and Methods

3.1 Materials

Brassica juncea with different leaf colors: including *B.juncea* (*lv*) (green leaf), *B.juncea* (*hong*) (lavender leaf), *B.juncea* (*zi*) (purple leaf). The leaves were provided by the Yan Mingli Laboratory of Hunan Science and Technology University and grew in the Biological Park of Hunan Science and Technology University. Primer synthesis, cloning and sequencing were provided by Tianyi Huiyuan Biotechnology Company. Plant DNA recovery and RNA extraction kits were purchased from Tiangen Biochemical Technology Co., Ltd. pMD18-T vector was purchased from TaKaRa company. *E. coli* DH5α strain, PCR and other reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd.

3.2 Determination of anthocyanin content in different leaf colors

Referring to Li et al. (2016), we took fresh leaves and dried them, then extracted 0.1 g of dried leaves and ground them into powder to extract anthocyanin. Using a UV spectrophotometer (Agilent Technologies Cary60 UV-Vis) to measure the absorbance at 530 nm, we calculated the total anthocyanin content in leaves by using the formula: anthocyanin content (mg/g)=A530×N×10×98.2-1.

3.3 Cloning of *PAP1* gene

The young leaves of *Brassica juncea* with different leaf colors (purple, red, green) at the three-leaf stage were collected, and the leaf DNA was extracted by the CTAB method. We took the published *PAP1* gene sequence (Bra039763; Bra001917; Bra004162) of *Brassica rapa* in the *Brassica* database (http://brassicadb.org/brad/) as a template, the primers PAP1.1 (Bra039763 homology) PAP1.2 (Bra001917 homology) PAP1.3 (Bra004162 homology) were designed online through the NCBI-BLAST website. The annealing temperature of the primer was set at 55℃ for 35 cycles. The PCR products were detected by 1% agarose gel electrophoresis, and the results were observed with a UV gel imaging analyzer. After the PCR product was purified by agarose gel DNA recovery kit, it was connected to the pMD18-T vector to transform the competent cells of *E. coli* DH5α. After resuscitation, plating and colony culture, we marked and picked the scattered and smooth single colonies, and used M13

universal primers for bacterial liquid PCR, the primer sequence (Table 3). After the positive clones were screened out, the positive clones were sent to the company for sequencing.

Table 3 Primer names and sequences for gene cloning and expression analysis

Name	Forward sequence	Reverse sequence	Purpose
PAP1.1	AACACTAATCAAGTTCTACAGTCT	TCTTTGGTCCATGGAGGATT	Homologous clone
PAP1.2	AAGAAACCAGGTACCTCCTAA	TAACACTAATCAAGTTCTACAGTCT	
PAP1.3	TTCCTCAAGCCTGCCTTTAC	ATGTCACGCACAAGCACAAA	
M13	CAGGAAACAGCTATGAC	GTAAAACGACGGCCAG	Colony PCR
qPAP1	GCTTTTAGGAAACAGGTTTG	TGAAGGATCGAGGTCGAGGT	qRT-PCR
qEGL	CTCTCTCCCTAGCGGAATCT	GCGAGAAGAGAGCGAGTGAA	
qGL3	CCAGCTAATCCTCGGACCAC	AGTTTCTCCCGCCGTTTCTT	
qTT8	TGGATACTACAACGGCGCAA	GGCATCCCAGAAGGAGGTTC	
qTT19	AAGGTGGTGATGTGTGGTGTG	GGCGTCACATTCTTCGCCTA	
qDFR	AAGAGACCGTGTGCGTAACC	GGCGTCATCGTAGCTTCCTT	
Actin	TCCATCCATCGTCCACAG	GCATCATCACAAGCATCCTT	qRT-PCR reference gene

3.4 Bioinformatic analysis of *PAP1* gene

We used DNAMAN v.8 software to splice the sequence. According to the *Brassica* database, we inferred the exon and intron regions of the sequence and translated it into a protein sequence to predict its isoelectric point. The online website ProtParam (https://web.expasy.org/protparam/) was used to analyze the amino acid composition and hydrophobicity of the protein sequence encoded by the *PAP1* gene. The MEGA7.0.14 software was used to compare the PAP1 protein sequence of *Brassica juncea* with PAP1 homologous sequences of other types of plants, and constructed a phylogenetic tree. Finally, we used PRABI, SWISS-MODEL, SMART and other websites to speculate on the secondary structure, tertiary structure and conserved domains of the protein encoded by the *PAP1* gene.

3.5 Expression analysis of *PAP1* gene

Taking the young leaves of *B.juncea* (*lv*) and *B.juncea* (*zi*) at the three-leaf stage, extracting the total RNA by using TRIzol kit, and removing impurity DNA by using RQ1 DNase (promega), we got purified RNA. Then the quality and concentration of purified RNA were measured under the absorbance of a UV spectrophotometer A260/A280. And its integrity was check by agarose gel electrophoresis. The obtained RNA was reverse transcribed with the ReverAid^{TM} First Strand cDNA Synthesis kit to obtain cDNAs in *B.juncea* (*lv*) and *B.juncea* (*zi*). Primer software was used to design suitable primers (Table 3), and the Actin gene was used as an internal reference primer for qRT-PCR amplification to perform gene expression analysis.

Authors' contributions

HD designed and executed the experiments. And she completed data analysis and wrote the first draft of the manuscript. ZWP, LW CJL and HP participated in designing the experiments and analyzing the results. ZDW conceived the project and was responsible for the project. And he directed the experiments, data analysis, paper writing and revision; YML participated in directing the experiment, paper writing and revision. All authors read and approved the final manuscript.

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