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Full Length Article

Functional Analysis of F3'5'H and Patterns in the Accumulation of Anthocyanin in Blackcurrant (*Ribes nigrum*)

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Abstract

In this study, the full-length cDNA sequence of the flavonoid 3′5′-hydroxylase (*F3'5'H*) gene of blackcurrant (*Ribes nigrum* L.) was cloned by reverse transcription polymerase chain reaction (RT-PCR) (GenBank accession number KC493688) to explore the expression and function of the *F3'5'H* gene in the synthesis of anthocyanins in this plant. First, the protein expressed by this gene was analyzed using bioinformatics tools to predict its function. Then, the expression of the *F3'5'H* gene in different tissues and organs of blackcurrant, such as the roots, stems, leaves, flowers, buds, and fruit at different stages of development was detected by quantitative PCR. In addition, high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) was performed for the qualitative and quantitative analysis of the anthocyanins in different plant parts and developmental periods. The complete open reading frame (ORF) of the cloned *F3'5'H* gene was ligated into the prokaryotic expression vector *pET28b* and transformed into three different strains of *Escherichia coli*. Next, the induction temperature and time for gene expression in the prokaryotic vector were optimized, and the expression of the gene product was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results illustrated that the cloned cDNA of the *F3'5'H* gene was 1530 bp in length and encoded 509 amino acids. The protein expressed by this gene had a relative molecular weight of 57.11 kDa and an isoelectric point of 9.26 and was a hydrophilic protein. This gene belongs to the CYP450 gene family and encodes no signal peptide. The expression level of *F3'5'H* was the lowest in flowers, and the highest in the fruit peel during the middle and late stages of fruit development, and then decreased afterwards. Analysis of the correlation between gene expression levels and the total anthocyanin content indicated that the two were significantly and positively correlated. The optimization results suggested that using *E. coli* strain No. 3, 16°C, and 16 h were the best induction conditions. Under these optimal conditions, a protein 57 kDa in mass was successfully expressed, which was in line with the expected size of the F3'5'H protein. Overall, this article establishes a foundation for further research investigating the relationship between F3'5H and anthocyanins in blackcurrant and will help researchers understand the important role of F3'5H in the synthesis of anthocyanins from blackcurrant.

Keywords: Cloning; *F3'5'H* gene; Prokaryotic expression; *Ribes nigrum*; Spatiotemporal expression

Introduction

Blackcurrant (*Ribes nigrum* L.) is a woody shrub native to high-latitude areas in the Northern Hemisphere that is cultivated in the three provinces of Northeast China (Xu *et al*. 2017). At present, no genomes of representatives of the genus *Ribes* have been sequenced or annotated. Anna V. Pikunova and colleagues have used DNA markers to extensively study blackcurrant, including by using amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), and single nucleotide polymorphism (SNP) markers to construct several genetic maps of linkage groups and locating a series of quantitative trait loci (QTLs) in this plant (Pikunova *et al*. 2019). Overall, molecular genetics studies of blackcurrant have been performed in a relatively backwards manner. It means that analyses typically done later in a genetics research program (more detailed/specific tests) have been done, but not more general/basic tests typically done earlier. Flavonoid hydroxylase reacts with different sites on molecules of naringenin. When flavanone 3-hydroxylase (F3H) is hydroxylated at the C3 position of naringenin, dihydrokaempferol (DHK) is formed, whereas when flavonoid 3′-hydroxylase (F3'H) is hydroxylated at the C3 position, eriodictyol is created, which is then is catalyzed by F3H to form dihydroquercetin (DHQ). When flavonoid 3′5′-hydroxylase (F3'5'H) is hydroxylated at the C3 and C5 positions, pentahydroxyflavanone is formed, which is then catalyzed by F3H to form dihydromyricetin (DHM). The substrate specificity and enzyme activity of F3H, F3'H, and F3'5'H differ among plant species, leading to variations in the amounts of DHK, DHQ, and DHM synthesized in them, and thus different anthocyanins being formed in different

plants (Liu *et al*. 2018). At present, few studies have been done on the molecular biology of blackcurrant, so the critical enzymes involved in the biosynthetic pathways of pigments in this plant still need to be investigated.

Anthocyanins are a class of flavonoids that are widely present in the roots, stems, leaves, flowers, and fruits of many plants and have significant effects on plant coloring. Delphinidin is an essential component of blue-violet pigments that is the product of a flavonoid biosynthetic pathway in which F3'5'H is the critical enzyme. In this pathway, F3'5'H competes with F3'H and flavonol synthase for substrates and catalyzes the 3'5'-end of compounds containing benzene rings, such as flavanone, eriodictyol, and DHK. It then forms dihydromyricetin, a direct precursor of blue-violet cyanins. Blue-violet cyanin pigments cannot be formed without this enzyme, so the gene encoding it is also referred to as the 'blue gene' of plants (Peng *et al*. 2019). Holton *et al.* (1993) were the first researchers to clone *F3'5'H* cDNA and did so from *Petunia* plants. *F3'5'H* cDNA was later cloned from *Gossypium* (used as a probe), *Pericallis cruenta*, and *Phalaenopsis* cDNA libraries (Whang *et al*. 2011; Ishiguro *et al*. 2012; Wang *et al*. 2014). In addition to coloring, anthocyanins can also affect seed transmission, and may also be used for human health, including for the improvement of vision and circulation, and for their anti-inflammatory, anti-oxidation, and anti-cardiovascular disease effects (Liang and Bao 2017).

The content of anthocyanins is the highest in the skin (peel) of blackcurrant fruits. The study of the blackcurrant *F3'5'H* gene is essential to improve our understanding of cyanidin biosynthesis and activity. Many studies have isolated and purified *F3'5'H* genes from plants, such as *Gossypium*, *Pericallis cruenta*, and *Phalaenopsis*, but such research has not been done on blackcurrant. In this study, reverse transcription polymerase chain reaction (RT-PCR) technology was used to clone the full-length cDNA sequence of the *F3'5'H* gene from the skin of blackcurrant, and this sequence was then analyzed by bioinformatics. In particular, real-time, fluorescence-based quantitative PCR was used to detect the expression of *F3'5'H* in different tissues and organs of blackcurrant, such as the roots, stems, leaves, flowers, buds, and fruits in various stages of development. This was done to test the correlation between the expression of *F3'5'H* and the content of anthocyanins in fruits. Simultaneously, a prokaryotic expression system was constructed for the *F3'5'H* gene. This study provides a theoretical basis for determining the role of the *F3'5'H* gene in cyanin biosynthesis in blackcurrant and developing methods to efficiently increase its expression.

Materials and Methods

Materials

Buds, flowers in full bloom, roots, young leaves at the tops of branches, old leaves at the bases of branches, annual stem segments of about 3–5 nodes in length, and pericarps at 20, 27, 34, 41, 48, 55, 62, and 69 days after flowering (DAF) were collected from blackcurrant shrubs in the present study. The blackcurrant variety 'Brodforp' was used as the source of these experimental plant materials. Samples were taken from 4-year-old field-planted fruit trees. The soil, fertilizer, water supply, and cold protection measures were consistent across trees and years. The plant materials were collected from the blackcurrant plantation resource garden at the Horticultural Experimental Station of Northeast Agricultural University, Harbin, China and were immediately frozen with liquid nitrogen and stored at -80°C until they were used in subsequent analyses.

Methods

Cloning of the blackcurrant *F3'5'H* gene: The modified CTAB (cetyl trimethylammonium bromide) method of Liu (2010) was employed to extract RNA from plant tissue samples. After extraction, analyses were done on 1% agarose gel to test RNA integrity, concentration and purity. RNA of sufficient quality was used as the template to synthesize cDNA with a TransGen Biotech kit, while following the manufacturer's instructions. Based on the nucleotide sequence and amino acid sequence of the *F3'5'H* gene and protein reported in GenBank (accession number KC493688), upstream and downstream primers (F1 and F2, respectively) for this gene were designed using Primer 5.0. The primers, whose sequences are reported in Table 1, were synthesized by Biotech (Shanghai) Co., Ltd. Additional *F3'5'H* gene primers were also used to obtain target gene fragments by PCR amplification. The PCR reaction conditions were as follows: pre-denaturation at 94°C for 5 min, followed by 94°C for 30 s; an annealing reaction at 52.6°C for 30 s and then 72°C for 1 min 40 s; extension for a total of 35 cycles; and a final extension step at 72°C for 10 min. The amplification procedure used is further detailed in Table 1. The target gene fragment was obtained by gel extraction using pre-designed kits, while referring to the kits' instruction manuals, and was then inserted into the clone vector to obtain the junction product, which was then used to transform *Escherichia coli* DH5 cells. Competent transformed *E. coli* cells positive for the cloned sequence were then used for sequencing.

Analysis of the cDNA sequence and amino acid sequence of the encoded protein: Full-length contigs were obtained after sequencing and were then subjected to sequence analysis using different bioinformatics tools (Table 2).

Spatiotemporal expression analysis of the *F3'5'H* gene in blackcurrant: Isolation of RNA from different parts of blackcurrant and the cloning of target genes followed the same specific procedures described above. Primers for quantitative RT-PCR were designed in accordance with the requirements of real-time fluorescent primers, and the *actin* gene was used as an internal reference to detect primer specificity. The primers used were synthesized by Biotech (Shanghai) Co., Ltd. The reaction conditions were as

Table 1: The DNA sequence and PCR amplification

Cloning of blackcurrant F3'5'H Gene	The primers sequences	F1: 5'-ATGGCGACCTTAGACATAATCCT-3'
		F2:5'-TTAAGCT TGATATGCAGAAAGAGC-3';
	amplification conditions	1.0 μL cDNA
		1.0 μL F1
		1.0 μL F2
		10 μL2×EasyTaq®PCRSuperMix
		7.0 μL ddH2O
	product size	1500bp
Spatiotemporal expression analysis of F3'5'H gene of blackcurrant	The primers sequences	FHF: GCGTGGATGGACTTACAAGGAAT; FHR: TGTCTGTACCCGCCGTAAATAAA; Actin F: CCGTCTCCAGAGTCCAGAACAATAC; Actin R: CTCACTGAAGCTCCTCTCAACCCAAAG
	amplification conditions	2.0 μL cDNA
		0.8 μL FH F/Actin F
		0.8 μL FH R/Actin R
		10 μL SYBR Premix Ex Taq II(Tli RNaseH Plus) (2×)
		6.4 μL ddH2O
	product size	100 bp-250bp
Prokaryotic expression of F3'5'H gene of blackcurrant	The primers sequences	M-F1: CCATGGATGGCGACCTTAGACATAATC (crossed for Nco Ienzyme loci)
		M-F2: CTCGAGTTAAGCTTGATATGCAGAAAGAG (Crossed for Nco IIenzyme loci)
	amplification conditions	Same as cloning of Blackcurrant F3'5'H Gene
	product size	1500 bp

Table 2: Sequence analysis software and sequence online analysis tools

Software	URL	Analysis of content
DANMAN		Amino acid function analysis
NPS@	https://blast.ncbi.nlm.nih.gov/Blast.cgi	Amino acid sequence analysis
Blastp	https://blast.ncbi.nlm.nih.gov/Blast.cgi	Amino acid homology analysis
ProtParam	https://web.expasy.org/protparam/	Protein molecular weight; isoelectric point; hydrophilicity, *etc.*
Signal P 4.0	https://www.cbs.dtu.dk/services/SignalP-4.0/	Signal peptide
HMMTOP	https://www.enzim.hu/hmmtop/html/submit.html	Transmembrane region
NPS@		Secondary structure of the protein
MEGA 7.0		Phylogenetic tree construction
PSORT 946 Prediction	https://psort1.hgc.jp/form.html	Subcellular localization analysis

follows: 94°C for 5 min; 94°C for 30 s; 57°C for 30 s; 72°C for 30 s; and 40 cycles of extension. The stability range of the dissolution curve was 55–95°C, and 3 biological replicates of each sample were tested. The reaction system is described in detail in Table 1.

Prokaryotic expression of the *F3'5'H* gene of blackcurrant: A pair of primers (Table 1) containing restriction sites were designed based on the *F3'5'H* open reading frame (ORF) sequence of blackcurrant and the multiple cloning sites of the prokaryotic expression vector *pET28b*. The targeted *F3'5'H* sequence of blackcurrant was amplified by the designed primers. The *F3'5'H* gene of blackcurrant and the *pET28b* prokaryotic expression vector were double-digested and then the target fragment was recovered with a gel recovery kit (TransGen Biotech). The digested target gene was then ligated with T4 ligase and the vector fragment. The recombinant plasmid *pET28b-F3'5'H* obtained was further transformed into competent DH5α cells. The optimal conditions for protein expression were selected to re-induce the appearance of the target protein. Moreover, the characteristics of the target protein were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In this analysis, the uninduced recombinant expression vector was used as the control group, and the recombinant protein concentration was measured using the Bradford method.

Qualitative and quantitative analyses of anthocyanins in blackcurrant: The plant samples were frozen in liquid nitrogen and then ground up into a powder. Afterwards, they were lyophilized in a lyophilizer for 24 h and stored at -80°C for later use. Malvidin-3-glucoside (1 mg) was dissolved and diluted to make a chromatographically pure fixed-volume solution. An anthocyanin standard curve between 1–80 mg/L was then established, with the average peak area (Y) set as the ordinate, and the concentration (X mg/L) set as the abscissa. The formula of the standard curve obtained was Y = 19988X - 17879, and the correlation coefficient thereof was 0.9989. The standard curve showed there was a good linear relationship between concentration and peak absorbance over the range of 1–80 mg/L (with concentrations converted into units of mg/g). Anthocyanins were extracted from samples according to the methods of Zhao (2017). Chromatographic analysis was conducted by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) under the following conditions: a Waters BEH C18 column (2.1 × 100 mm, 1.7 μm particle size) was used; the column temperature was 25°C; the flow rate was 0.3 mL/min; the injection volume was 5 μL; and the detection wavelength used was 525 nm. The mass spectrometry analysis was carried out using an ESI source as the ion source, in the positive ion detection mode. The spray voltage (ISVF) was set to 5500 V, and the ion source temperature (TEM) was 600°C. Each sample was analyzed in triplicate.

Statistical analyses

The data were read automatically by fluorescence quantitative PCR. According to the formula $Q = E^{-\Delta\Delta Ct}$, he relative level of expression of the *F3'5'H* gene in different tissue parts and at different fruit developmental stages of the 'Brodforp' variety of blackcurrant was calculated. In this formula, E is the efficiency of amplification (the default value was set at 2), and $\triangle\triangle C_t = \triangle Ct(sample) - \triangle C_t(contrast)$. The formula of the standard curve discussed above (Y = 19988X - 17879) was used to calculate the content of anthocyanins in different tissue parts and at different fruit developmental stages. Based on the peak areas of the mass spectra obtained by HPLC-MS/MS, the content of six major anthocyanins in blackcurrant parts was calculated. The experimental data were subjected to statistical analyses in Microsoft Excel and DPS 7.0 software (https://www.chinadps.net/), and charts were produced with Origin 8.0 (OriginLab). All data are presented herein as the mean ± standard error (SE) calculated across three replicates.

Results

Synthesis of the full-length cDNA sequence of the *F3'5'H* gene of blackcurrant

The improved CTAB method was successfully used to extract RNA from blackcurrant, and no visible RNA degradation occurred. In this study, cDNA was obtained by reverse transcription, and the target fragment was amplified using the primers F1 and F2. Agarose gel electrophoresis detected a band of 1500 bp in size, which was similar to the expected fragment size (Fig. 1).

The product was ligated and transformed into a prokaryotic vector, and 15 single colonies were randomly selected for enumerating. As shown in Fig. 2, 11 colonies were amplified by PCR to produce a 1500 bp band, verifying that they were positive for the target fragment and that the gene of interest had been successfully ligated into the *pMD18-T* cloning vector. The liquid media of five test tubes containing positive prokaryotic cloning vector colonies were randomly selected for sequencing to identify the complete ORF sequence of the target gene. The result was compared with the registered blackcurrant *F3'5'H* gene in GenBank and found to have a homology of 99.41% with it.

Bioinformatics analysis of *F3'5'H*

The results of ORF analysis showed that the sequenced gene was 1530 bp in length. The conservative protein motifs in the product of the *F3'5'H* gene were predicted by performing BLAST searches of the National Center for Biotechnology Information (NCBI) database. As a result, this gene was found to belong to the cytochrome P450 supergene family. Software analysis indicated that the gene

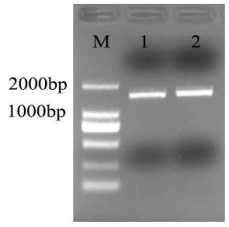

Fig. 1: The ORF amplification of F3'5'H
Agarose gel electrophoresis was detected by gel imager. The RT-PCR products of F3'5'H primers are band 1 and band 2 respectively. M is Trans 2000 DNA maker

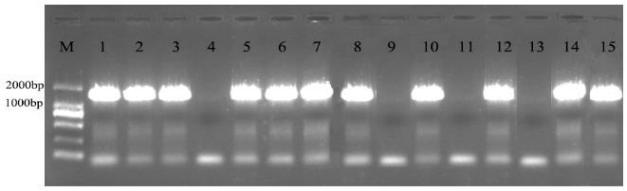

Fig. 2: Bacterial solution PCR verification
The result of 15 random single colonies was shown, 11 of which were positive. M is Trans 2000 DNA maker.

encoded a total of 509 amino acids, comprising a protein with a theoretical molecular weight (MW) of 57.11 kDa and a theoretical isoelectric point (pI) of 9.26. F3'5'H signal peptide prediction results indicated that the gene did not contain a signal peptide, suggesting that the protein encoded by this gene was not a secreted protein or a membrane protein. The ratio of sparseness to hydrophilicity was between -3.467 and +2.578 for the encoded protein, meaning that the number of hydrophilic amino acids in the protein was higher than that of hydrophobic amino acids. The protein encoded by this gene was speculated to be a hydrophilic protein, where regions 1–29 of the protein had durable hydrophobicity. Therefore, the hydrophobic part of the blackcurrant F3'5'H protein is most likely a transmembrane component. The blackcurrant F3'5'H protein was predicted to have a transmembrane helix located at regions 12–31 at its N-terminus. Subcellular localization analysis predicted that the protein was the most likely to be localized in the cytoplasm, with a probability of 39.1%. The likelihood of localization to the mitochondria and the nucleus was 17.4%, the possibility of localization to the endoplasmic reticulum was 13.0%, and the likelihood of localization to the Golgi apparatus was 4.3%. The predicted secondary structure of the protein is displayed in Fig. 3. The encoded protein was predicted to contain: 255 alpha-helixes, accounting for 50.10% of its length; 50 extended strands, accounting for 9.82% of the protein; and 204 random coils, accounting for 40.08% of the protein.

MEGA7.0 and phylogenetic tree analyses were used to compare the sequence of this gene's product with the amino acid sequences encoded by the *F3'5'H* genes of other species (Fig. 4). The amino acid sequence encoded by the *F3'5'H* gene of *Vitis vinifera* was the closest to that found for blackcurrant herein, although the protein of *V. vinifera* was

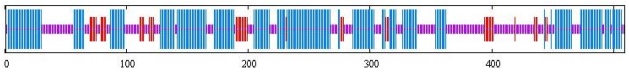

Fig. 3: Prediction of the secondary structure of F3'5'H was shown. There were 255 alpha-helixes, 50 extended strands and 204 random coils in the encoded protein.
Alpha helix: blue; Extended strand: red: Beta turn: green; Random coil: purple

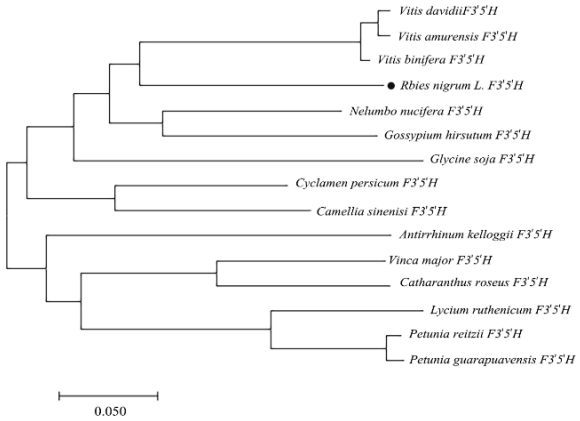

Fig. 4: A phylogenetic tree inferred using MEGA 7.0 from the deduced amino acid sequences for *F3'5'H*

the most closely related to that of *Petunia guarapuavensis*.

Analysis of spatiotemporal patterns in the expression of *F3'5'H*

Patterns in the expression of the *F3'5'H* gene in the roots, stems, flowers, buds, young leaves, old leaves, and fruits of blackcurrant at different stages of development were detected by real-time quantitative PCR. The results (Fig. 5) indicated that *F3'5'H* was expressed in all tissues and organs, but its expression levels were significantly different among organs and fruit developmental stages. The lowest expression levels were detected in flowers, and the highest expression levels were detected in fruit peels. Before the fruit was fully mature, the expression level of the *F3'5'H* gene gradually increased as the degree of coloring of the fruit increased. Its expression was the highest during the ripening period (9 weeks after flowering (WAF), when the fruit coloring rate was 80–90%), and then decreased sharply after this, reaching the lowest expression levels at the late stage of fruit maturity.

Qualitative and quantitative analyses of anthocyanins during the development of blackcurrant

Six major anthocyanins were detected in different tissue parts and different fruit developmental stages of the 'Brodforp' variety of blackcurrant by HPLC-MS/MS, as shown in Fig. 6. Among these pigments, the content of delphinidin was the highest, and it gradually increased with the development of the fruit, reaching a maximum value of 16.00 mg/g at 9 WAF and then decreasing in the peel of the fully mature fruit. The second highest content was that of anthocyanin, which was up to 12.6 mg/g in fully ripe fruit skins. Methyl-delphinidin, methyl-anthocyanin, dimethyl-delphinidin, and pelargonidin could be detected at 6 WAF, and then the content of these pigments gradually increased until the fruit was fully mature, reaching peak values of 3.75, 3.45, 2.98, and 3.39 mg/g, respectively. However, these four pigments were not observed in the roots, stems, leaves, flowers, or buds of blackcurrant.

As illustrated in Fig. 7, the content of anthocyanins in plants parts other than the fruit peel was low. The total content of anthocyanins in the fruit peel at 6 WAF was 11.95 mg/g, which was double that at 3–5 WAF. It then increased to 15.38, 24.67, and 34.75 mg/g at 7, 8, and 9 WAF, respectively, and was 37.25 mg/g in the late mature fruit. In summary, the content of anthocyanins in the peel of blackcurrant fruits is higher than that in any other tissues and organs of this plant, and gradually increased during the process of fruit development up to the point at which fruits reached full maturity.

Correlation between anthocyanin content and *F3'5'H* gene expression during the development of blackcurrant

From the results of the correlation analysis performed (Fig. 8), it can be seen that the relative expression level of the *F3'5'H* gene was positively correlated with anthocyanin content. The expression level of this gene in the fruit peel was higher than that in all other tissues and organs, and gradually increased with fruit development. The expression level of the *F3'5'H* gene considerably decreased during the ripening period of the fruit, while the content of anthocyanins still increased over this period.

The results of analyses of the correlation between the total amounts of six anthocyanins in blackcurrant and the relative expression levels of the *F3'5'H* gene are presented in Fig. 9a–f. It can be seen that the relative expression level of the *F3'5'H* gene was the most strongly correlated with the content of delphinidin, with there being a clear positive relationship between these two variables (Fig. 9a). The expression of this gene was the most weakly associated with the content of methyl anthocyanin in blackcurrant.

Prokaryotic expression of *F3'5'H*

The recombinant plasmid *pET28b-F3'5'H* was transferred into *E. coli* strain No. 1, strain No. 2, and strain No. 3. A set of empty vector strains were also established as a control group. Expression of the target gene was induced using 1 mg of isopropyl β- d-1-thiogalactopyranoside (IPTG) under different temperature conditions of 16, 30, and 37°C, and using different induction times. Samples were then centrifugated, and the resultant supernatant was then precipitated and analyzed by SDS-PAGE. The expression

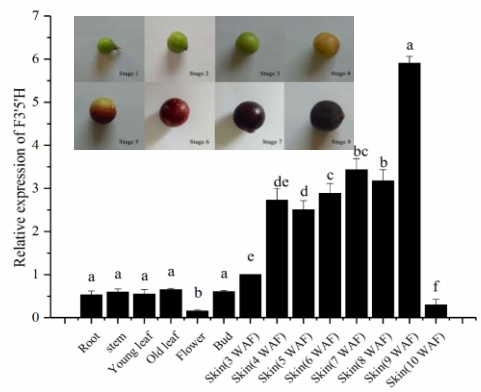

Fig. 5: The relative expression of the F3'5'H gene in different tissues and fruits of *R. nigrum*

Histograms with different letters indicate a significant difference in gene expression ($P < 0.05$); WAF: weeks after flowering

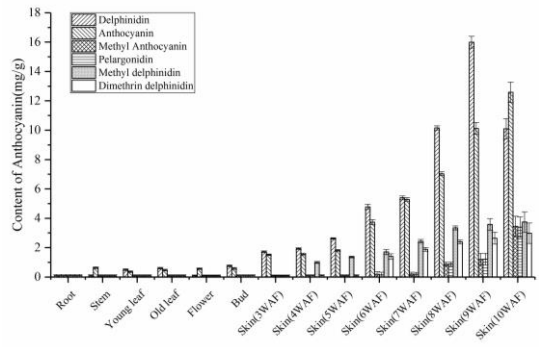

Fig. 6: The content of various anthocyanins in different tissues and fruits of *R. nigrum*

levels of the target protein in different *E. coli* strains are shown in Fig. 10. Target protein expression in *E. coli* strain No. 3 was significantly higher than that in the other two strains and increased as the temperature decreased. F3'5'H expression in all three strains indicated that the target protein was mainly expressed when expression was induced at high temperatures, and the protein was found in the precipitate formed after sonication in the form of inclusion bodies. A soluble protein was also detected in the ultrasonic supernatant when expression was induced at low temperatures. The recombinant protein pET28b-F3'5'H was successfully expressed in the form of a soluble protein in the tested *E. coli* expression system. A specific protein expression band could be observed around 57 kDa, which was close to the predicted size (approximately 57 kDa) of the target protein.

After screening, the best conditions for inducing *F3'5'H* prokaryotic expression were using the *pET28b* vector in *E. coli* strain No. 3, with induction by 1.0 m*M* IPTG at 16°C and for 16 h. The concentration of purified blackcurrant F3'5'H enzyme protein induced was determined by the Bradford method; the standard curve equation was: $Y = 3.238X + 0.6759$ ($R^2 = 0.9814$). The protein concentration under optimal expression conditions

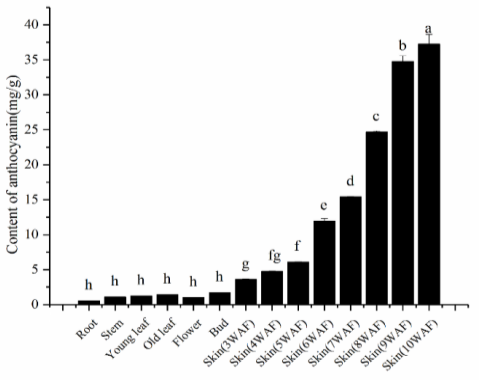

Fig. 7: The content of anthocyanins in different tissues and fruits of *R. nigrum*

Histograms with different letters indicate a significant difference in gene expression ($P < 0.05$)

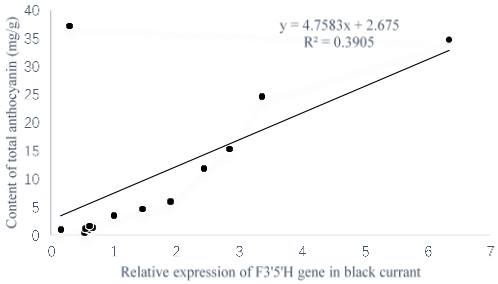

Fig. 8: Correlation between relative expression of F3'5'H gene and total anthocyanin content in *R. nigrum*

calculated from this equation was 0.41 mg/mL.

Discussion

In the conserved motif of the F3'5'H protein, the amino acid sequence 'PPGP' appears to be used to connect the anchor site of the cell membrane to the sphere of the enzyme protein. The formation of a binding region for oxygen molecules and proton transfer is promoted at the 'AGTDT' amino acid sequence, which is related to binding; indeed, the similar 'FGAGRRICAG' sequence was previously found to be related to heme binding (Seitz *et al*. 2015). The amino acid sequences of the F3'5'H proteins of different species mainly differ in a region of about 31 amino acids at the N-terminus (Jia *et al*. 2019). Comparisons with the homologous proteins of other species (Fig. 4) indicated that the blackcurrant F3'5'H was the most closely related to those of grapevines (*Vitis* spp.), such as grapevine and spiny grape, followed by those of other dicotyledonous plants (water lotus and cotton), which was in accordance with the morphological classification of these plants. Therefore, it is feasible to use bioinformatics to speculate on the phylogenetic relationships among genetic species.

Bioinformatics analysis revealed that, in addition to the above highly conserved motif of the F3'5'H protein, there are other conserved regions with unknown functions.

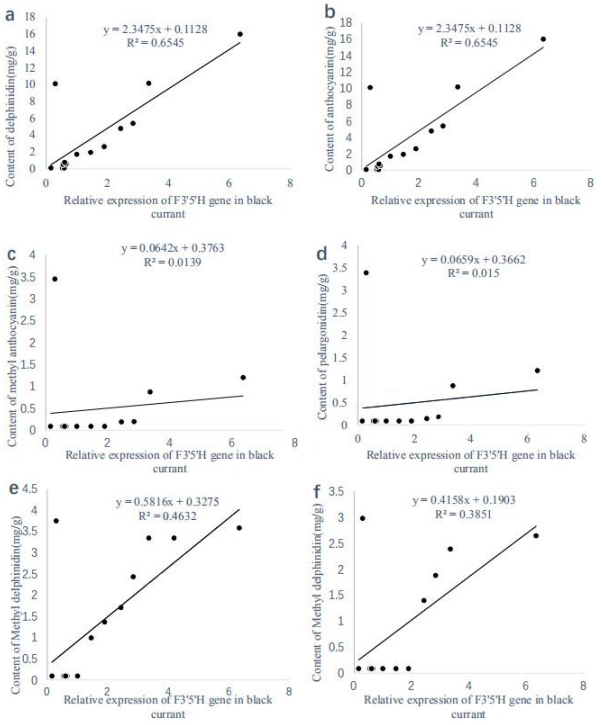

Fig. 9: Correlation between relative expression of F3'5'H gene and six kinds of anthocyanin content in *Ribes nigrum* L.

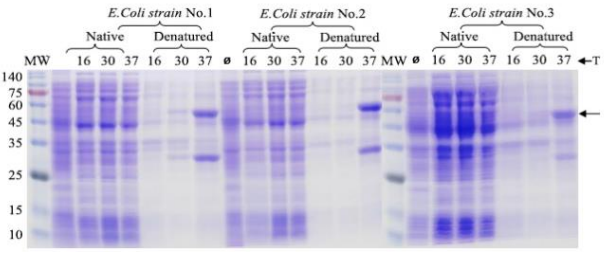

Fig. 10: Expression of pET 28b-F3'5'H in *E. coli*
MW: Molecular weight maker; ø; Non-induced bacteria culture (negative control); Native: Supernatant after sonication; Denatured: Pellet after sonication; T: Temperature; The induced proteins are arrowed

These regions also participate in the isolation of the *F3'5'H* gene of blackcurrant and may play important roles in its function. This suggests that determining the functions of these regions could help us to fully understand the mechanism of substrate competition between F3'5'H and other enzymes (Zhang *et al*. 2015). We also found that the F3'5'H enzyme protein mainly exists in the form of inclusion bodies in prokaryotic expression vectors. Previous studies showed that many exogenous proteins were produced primarily in the cytoplasm in the form of inactive inclusion bodies (Zhang *et al*. 2012). The reasons for this might be that the rapid and efficient expression of foreign genes prevents *E. coli* from secreting many enzymes and cofactors in time for protein processing, making the proteins produced unable to fold or form their correct secondary structure or bonds, resulting in a large number of proteins existing in the form of inclusion bodies (Park *et al*. 2012; Chuang *et al*. 2016; Guan *et al*. 2016) investigated the expression of the pomegranate *F3'5'H* gene under different temperature treatments by RT-PCR, and found that expression levels increased as temperature increased from 0 to 10°C. A better *F3'5'H* prokaryotic gene expression system was found herein by using different *E. coli* strains, temperatures, and induction times. As shown in Fig. 10, the protein concentration obtained was the highest using *E. coli* strain No. 3 and inducing expression with 1.0 m*M* IPTG at 16°C and for 16 h.

Our experimental results indicated that the expression of the *F3'5'H* gene exhibits significant spatial and temporal differences in blackcurrant. Huang and Fan (2012) explored the expression of the *F3'5'H* gene in *Strelitzia reginae*, and found that it began to be expressed at the flower bud stage, and then its expression changed over the course of flower development. Specifically, it gradually increased until the initial flowering period, when it was expressed at the highest levels observed, and then its expression began to decrease as the flowering period continued (Huang and Fan 2012); this is consistent with the results observed in the present study. The late fruit ripening stage (10 WAF) had the highest degree of fruit coloration and the lowest *F3'5'H* expression herein, indicating that the studied gene is not related to the synthesis of anthocyanins in the late fruit development period. The same phenomenon was found in a study by Xiao *et al.* (2015) done on the *F3'5'H* gene of colored potato. The possible reasons for the above phenomenon may be that either: (1) the accumulation of anthocyanin synthesis precursors at the later stage of fruit ripening has reached the highest level possible, and thus anthocyanins can be synthesized without the participation of this gene; or (2) the later stages of fruit development may involve the processes of anthocyanin methylation and anthocyanin glycosylation, and these changes do not require the participation of this gene. In one past study, the *F3'5'H* gene of *Dendrobium moniliforme* was found to be expressed at the highest levels at the base of the style, rather than in the calyx and petals (Sung *et al*. 2011). In the present study, the expression of the *F3'5'H* gene was also detected in parts of blackcurrant other than the fruit peel, although the expression levels therein were very low. This may be because one or more of the *F3'5'H* alleles expressed in these parts underwent multiple protein mutations, which hindered its expression (Xue *et al*. 2018). In some cases, functional and non-functional alleles of *F3'5'H* differ due to mutations affecting protein function rather than those affecting gene transcription. The leaves, stems, and buds of blackcurrant are green, the roots are brown, and the flowers are white, and the expression of the *F3'5'H* gene has some correlation with the color of plant parts. We detected 17 kinds of anthocyanins in different parts of blackcurrant by HPLC-MS/MS. The content of anthocyanins varied greatly among different plant parts, and the content of delphinidin was found to be the highest of all anthocyanins. Borges *et al*.

(2010) detected 12 anthocyanins in mature blackcurrant fruits. Svetlana *et al.* (2017) found that the content of anthocyanins in ripe fruits was about 141 times that in leaves. Lee *et al.* (2016) analyzed the cyanins in blackcurrant and found that delphinidin accounted for one-half of the total anthocyanin content therein. There was a positive correlation between *mir858* expression and anthocyanin content in 'Heijingang' and 'Huasong 66' purple potato varieties in a study by Fan *et al*. (2019), and a negative correlation was found between *mir858* expression and anthocyanin content in 'Hongmei' red potato varieties. In this study, the expression of *F3'5'H* in the 'Brodforp' variety of blackcurrant was positively correlated with its anthocyanin content, but this does not mean that the expression of *F3'5'H* in all blackcurrant cultivars is positively correlated with their anthocyanin content. Given the lack of research on the *F3'5'H* gene of blackcurrant, whether the specific variety of blackcurrant considered leads to differences in gene expression and anthocyanin regulation remains to be further studied.

Conclusion

At present, there have been few studies done on the genes of blackcurrant. In this study, the *F3'5'H* gene of blackcurrant was successfully cloned using homologous cloning technology. The results showed that the F3'5'H protein of blackcurrant has three CYP450-specific conserved amino acid sequences (FGAGRRICAG, PPGP, and AGTDT), and has high similarity with the F3'5'H protein of grape. The expression levels of this gene increased with the anthocyanin content in blackcurrant fruit peels, which suggested that this gene might be involved in the biosynthesis of anthocyanins in blackcurrant. These results provide reference data that can be used for the further exploration of the effect of F3'5'H on blackcurrant and contribute to the molecular breeding of this plant.

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