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ARTICLE

Genome-Wide Analysis of the *F3′5′H* Gene Family in Blueberry (*Vaccinium corymbosum* L.) Provides Insights into the Regulation of Anthocyanin Biosynthesis

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ABSTRACT

The F3′5′H gene family plays an important role in regulating anthocyanin biosynthesis, abiotic stress, and hormone signaling. In this study, 14 *F3′5′H* genes were identified from the blueberry genome. The chromosomal distribution, physicochemical properties, F3′5′H domain, conserved motifs, cis-acting elements, and intron/exon compositions were analyzed. The functional prediction analysis of these *VcF3′5′Hs* indicated that their biological functions included light response and other secondary metabolites. The results of qRT-PCR showed that *VcF3′5′Hs* (especially *VcF3′5′H4*) were highly expressed at the ripening stage. Subcellular localization revealed that *VcF3′5′H4* may be located in the endoplasmic reticulum. Co-expression analysis showed that the *VcF3′5′H* gene family was related to anthocyanin. This research provides an overview of the blueberry *F3′5′H* family and helps verify the role of these genes in regulating anthocyanin biosynthesis.

KEYWORDS

Blueberry; *F3′5′H*; anthocyanin; gene expression; co-expression

1 Introduction

The blueberry bush is a commercially significant shrub in North America and a popular fruit tree. Blueberry refers to the blue fruit produced by member species within the genus *Vaccinium* (Ericaceae). In China, 347,200 tons of blueberries were harvested from 66,400 hm^2 of cultivated area [1]. Guizhou Province has a blueberry planting area of 15,000 hm^2, with an annual output of 85,000 tons [1]. A growing number of studies have demonstrated the positive effects of fruit metabolites (e.g., anthocyanins) on human health, which drives the market expansion of this so-called "superfood" [2,3]. Anthocyanins are used to improve visual acuity and immunity, and have certain anti-inflammatory, anti-ultraviolet radiation, anti-pathogen infection, anti-aging, and anti-cancer effects [4–7]. On this basis, the economic value of blueberries can be improved by increasing their anthocyanin contents.

The blueberry anthocyanin synthesis pathway has been studied [8]. The purple pigment in blueberries is anthocyanin, mainly consisting of delphinidin-3-*O*-arabinoside [9,10]. Our preliminary research discovered that delphinidin accumulation is associated with the *VcF3′5′Hs* gene, which encodes the flavonoid 3′5′-hydroxylase (F3′5′H) enzyme [11]. In the anthocyanin metabolic pathway, F3′5′H, belonging to the cytochrome P450 (CYP) enzyme family, is the main enzyme in the synthesis of blue delphinium and crystalizes the hydroxylation of the 3′ and 5′ positions of the B ring of dihydroflavonols [12]. *F3′5′H* is also known as the blue gene, and its gram long offers the possibility of generating blue pigment [13]. In this sense, *F3′5′H* is thought to be the major enzyme gene that regulates blueberry anthocyanin production.

F3′5′H was first cloned from petunia and was found to be involved in pigment synthesis [14,15]. Its homologs were subsequently cloned from various plants, including *gentian* [16], *Arabidopsis* [17], *Solanum tuberosum* [18], *tomato* [19], *Ginkgo* [20], and *Aconitum* [21]. *F3′5′H* genes are linked to anthocyanin synthesis. The overexpression of the *F3′5′H* gene can promote the accumulation of poplar anthocyanins and enhance the hydroxylation of flavonoids [22]. The hydroxylation pattern of flavonoids is largely determined by *F3′5′H* genes. For example, cloning the *F3′5′H* genes can change the color of roses, carnations, and other plants without *F3′5′H* activity from lavender to blue [23–25]. Some *F3′5′H* genes have been used in breeding new varieties of blue ornamental plants [16,26,27]. In sum, the cloning and characterization of the *F3′5′H* gene are of special significance.

In the past few decades, many studies on anthocyanin biosynthesis have given an understanding of the F3′5′H pathway. Anthocyanins are natural water-soluble secondary metabolites belonging to the flavonoid metabolism branch of the phenylpropanoid pathway [28]. Many genes involved in this pathway, such as phenylalanine ammonia-lyase, 4-coumaroyl: CoA ligase, and chalcone synthase (CHS), have been identified. However, few studies have been conducted to analyze the *F3′5′H* gene family associated with blueberry anthocyanin biosynthesis. The biosynthetic pathway is relatively conserved among different species, and the genes regulating anthocyanin biosynthesis differ in organs and tissues. The *MYB* gene appears to play a key role in regulating anthocyanin biosynthesis. In blueberries, *VcMYBPA1.1* can activate *VcCHS*, *VcF3′5′H*, *VcDFR*, *VcANS*, and *VcGST* promoters and facilitate the anthocyanin synthesis [29]. Silencing *VmMYBPA1.1* in bilberry results in the decreased expression of *VmCHS*, *VmF3′5′H*, *VmANS*, *VmLAR1a*, and anthocyanin loss [30]. The R2R3-MYB transcription factor *SmMYB35* can bind to promoters, such as *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS*, to enhance their activity to promote anthocyanin synthesis in eggplant [31]. Studies have shown that the *F3′5′H* gene is regulated by MYB or bHLH transcription factors, thereby promoting anthocyanin synthesis. The study of the blueberry *F3′5′H* gene family is significant for the cultivation of blueberry varieties with high anthocyanin contents.

Blueberry (*Vaccinium* spp.) is one of the most important economic berries in the world. The finding of the whole blueberry genome sequence enables the performance of whole-genome *F3′5′H* gene analysis [32]. *F3′5′H* family research showed that *F3′5′H* members are related to anthocyanin biosynthesis, abiotic stress, and hormone signaling [10,33,34]. Anthocyanin accumulation at different developmental stages of blueberries resulted in color-phenotype differentiation. However, few studies have explored the genetic basis of the peel and pulp color. The genetic basis of anthocyanin regulation in blueberries can be further understood by combining phenotypic, genomic, and transcriptome analyses. In this study, a genome-wide analysis of *F3′5′H* in blueberries was performed, and 14 *F3′5′H* genes were identified. A comprehensive investigation of the gene structure, cis-acting element analysis, and conserved domains provided evidence for the functional annotation. Furthermore, subcellular localization and gene expression analyses indicate that the *VcF3′5′H4* gene is crucial in regulating blueberry anthocyanin synthesis. This study lays a foundation for exploring the role of the *F3′5′H* gene in anthocyanin biosynthesis in blueberries and promotes further functional characterization of these regulators.

2 Materials & Methods

2.1 Plant Materials

Five-year-old rabbiteye blueberries 'fenlan' (*Vaccinium ashei: Reade*) were grown in Guiyang, Guizhou, China, with a planting distance of 30 cm × 30 cm. The growing region was located at 106°27′−106°52′ east longitude and 26°11′−26°34′ north latitude. Three plants of uniform growth were selected for each treatment as biological replicates. Fruit samples were collected at 80 d (S1), 95 d (S2), and 110 d (S3) after the flowering (Fig. 1). All samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Figure 1: Fruit sampling period

2.2 Identification of *VcF3′5′H* Members and Phylogenetic Analysis

The blueberry genome file was aligned with the F3′5′H sequence mentioned by Liu et al. [35]. The alignment method was BLAST with an E-value of 1.0×10^{-5}, and 17 *VcF3′5′H* were identified by default parameter settings. P450 database download site was as follows: https://drnelson.uthsc.edu/. All protein sequences were aligned by the ClustalW program in MEGA 7, and then NJ phylogenetic tree was constructed based on the Poisson model with a bootstrap value of 1,000.

2.3 Motif Identification, Gene Structures, and Protein Motif Compositions of *VcF3′5′H* Members

The gene structures of 14 blueberry *F3′5′H* genes were extracted from the gff3 structural annotation file by Perl script. Online tools were employed for analysis. Specifically, GSDS 2.0 (http://gsds.cbi.pku.edu.cn/) was used for domain visualization [36], and meme (http://memesuite.org/) [37] was adopted for comparing the *VcF3′5′H* conserved motif with 10 conserved motif parameters and other default parameters; TBtools (https://github.com/CJ-Chen/TBtools) was used for motif visualization analysis [38], and molecular weight, stability coefficient, and hydrophobicity were analyzed using ExPASy [39].

2.4 Sequence Alignments, Chromosome Mapping, Profiles of *VcF3′5′H* Conserved Amino Acids, and Collinear Analysis of Anthocyanin

SOPMA was used to predict the secondary structure of *VcF3′5′H* family proteins. DNAMAN was employed for the protein alignment of the identified 14 *VcF3′5′H* genes. The structure files of *F3′5′H* genes in blueberries were extracted by TBtools [39] to determine their starting and ending positions in the chromosomes. Co-expression network analysis was performed on the *VcF3′5′H* gene and anthocyanins. The analysis tool was Cytoscape 3.7.2, and $R \geq 0.9$ was considered significantly correlated [40]. Anthocyanins were detected using LC-MS/MS. All data are shown in Supplementary Table S1.

2.5 Cis-Acting Elements Located in *VcF3′5′H* Gene Promoters

A 2000 bp upstream sequence of the gene was extracted by Bedtools [41], and was analyzed by the online tool PlantCARE [42], and the online tool GSDS 2.0 was used for the visual analysis of functional elements.

2.6 qRT-PCR

A total of 14 *VcF3′5′Hs* samples at three periods (S1, S2, and S3) were selected for qRT-PCR analysis. The primers for the genes were designed using Primer Premier 5.0. Total RNA was isolated from green, pink, and purple fruits by CTAB, and was checked on 1.2% agarose gel under UV light without smearing before the concentration detection using spectrophotometry. One microgram of total RNA was used to synthesize cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Japan) following the manufacturer's instructions. The primers were designed for PCR amplification (Supplementary Table S2). QRT-PCR was performed in a real-time PCR system (QuantStudio 3D digital PCR system, ABI) using SYBR Real Master Mix (TransGen, Beijing, China) under the following PCR thermal cycling conditions: pre-denaturation at 95°C for 30 s, and 40 cycles at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s, respectively. *GAPDH* (AY123769) bilberry genes were used as the housekeeping genes. Tree biological replicates were performed for each gene, and the standard curve method was applied in statistical analysis. The relative mRNA expression data were analyzed using $2^{-\Delta\Delta Ct}$ [43]. Excel v. 2010 (Microsoft Corp., Redmond, WA, USA) and SPSS v. 25 (IBM Corp., USA) program was used to analyze the data using one-way analysis of variance (ANOVA). Duncan's new multiple range test was used to separate means where ANOVA indicated significant differences at $p < 0.05$.

2.7 VcF3′5′H 4 Gene Cloning and Subcellular Localization Analysis

The CDS of the *VcF3′5′H4* gene was obtained by searching the reference genome (http://gigadb.org/dataset/100537) according to the gene number, and primers were designed for PCR amplification (Supplementary Table S3). PCR products were cloned into a pBWA(V)HS-ccdb-GLosgfp and sequenced (Sangon Biotech, Shanghai, China).

The *VcF3′5′H4* CDS with Bsal sites but without termination codons were amplified from the pBWA(V)HS-ccdb-GLosgfp plasmid template with Taq DNA polymerase (TaKaRa Biotechnology Co., Ltd., Japan). It was then inserted immediately upstream of the green fluorescent protein (GFP) coding sequence, and framed in the pBWA(V)HS-35s-GLosgfp vector, which had been digested with Bsal to generate pBWA(V)HS-*VcF3′5′H4*-GLosgfp vector with the cauliflower mosaic virus 35S promoter. The construct was subsequently introduced into *Agrobacterium tumefaciens* strain GV3101. To investigate the subcellular localization of *VcF3′5′H4* protein, 35S: *VcF3′5′H4*-GFP and negative control 35S: GFP plasmids were transformed into N. *benthamiana* as described previously (Johansen and Carrington, 2001) and cultured in a growth chamber for 48 h (25°C, 16 h light/8 h dark). Fluorescence visualization of GFP was performed using a Zeiss confocal microscope (LSM510; Carl Zeiss, Thornwood, NY, USA) with an excitation wavelength of 480 ± 20 nm and an emission wavelength of 510 ± 20 nm.

3 Results

3.1 Identification, Conserved Domain, and Gene Structure Analysis of the VcF3′5′H Gene Family

A total of 17 blueberry *VcF3′5′H* genes were identified using the BLAST alignment with reference to the *F3′5′H* gene sequence provided by Liu et al. [35] (Supplementary Figure S1). Only 14 *F3′5′H* sequences and a CYP75 subtribe were discovered to be together when we examined 17 samples with P450 subfamily sequences for an evolutionary study (Supplementary Figure S1). KEGG annotation was used to further verify that the 14 genes were blueberry *F3′5′H* genes. All of them were mapped to pseudochromosomes (*VaccDscaff*) and renamed from *VcF3′5′H1* to *VcF3′5′H14* according to the order of locations on the pseudochromosomes. Gene characteristics were analyzed (Table 1). The result showed that the shortest proteins were *VcF3′5′H11*, *VcF3′5′H13*, and *VcF3′5′H14* (508 amino acids), and the longest one was *VcF3′5′H1* (601 amino acids). According to the analysis, the molecular weights of the 14 *VcF3′5′H* proteins ranged from 56.7 to 66.4 kDa, and the isoelectric points ranged from 7.14 to 9.19. The hydrophilicity coefficient was between −0.207 and 0.053. Except for *VcF3′5′H1*, *VcF3′5′H6*, and

VcF3′5′H11, which were hydrophobins, other family members were hydrophilic proteins. Secondary structure prediction revealed that the secondary structures of the *VcF3′5′H* gene family were dominated by the α-helix, ranging from 41.73% to 46.09%, followed by the random coil (30.27%–33.07%); the extended strand accounted for 14.17%–17.58%, and the β-turn was relatively less (7.32%–10.83%). As shown in Fig. 2C, the numbers of exons in *VcF3′5′H* genes were between 2 and 4, with an average of 2.4. Among all *VcF3′5′H* genes, 11 contained two exons and accounted for approximately 65% of *VcF3′5′H* gene family members, whereas only 3% of *VcF3′5′H* genes had more than three exons (Fig. 2C). The differences in protein sequences among the blueberry *VcF3′5′H* were analyzed using Multiple Expectation Maximization for Motif Elicitation online tool.

Table 1: Physical and chemical properties of blueberry F3′5′H proteins

Number	Gene name	Amino acids	Molecular mass (Da)	Isoelectric point	Hydrophilic	α-helix (%)	Extend strand (%)	Random coil (%)	β-turn (%)
1	*VcF3′5′H1*	601	66384.99	9.19	0.053	43.93%	15.97%	32.78%	7.32%
2	*VcF3′5′H2*	512	56777.31	8.61	−0.032	44.34%	16.80%	30.86%	8.01%
3	*VcF3′5′H3*	512	56788.27	8.6	−0.058	43.55%	17.38%	31.05%	8.01%
4	*VcF3′5′H4*	512	56802.39	8.84	−0.044	46.09%	15.23%	30.47%	8.20%
5	*VcF3′5′H5*	512	56738.17	8.43	−0.041	44.14%	17.58%	30.27%	8.01%
6	*VcF3′5′H6*	537	59649.87	8.88	0.017	44.13%	17.32%	30.35%	8.19%
7	*VcF3′5′H7*	512	56880.47	8.77	−0.019	43.36%	17.19%	31.45%	8.01%
8	*VcF3′5′H8*	512	57136.66	8.74	−0.066	45.70%	15.43%	30.86%	8.01%
9	*VcF3′5′H9*	512	57150.73	8.73	−0.065	46.09%	15.43%	30.27%	8.20%
10	*VcF3′5′H10*	510	57155.65	8.39	−0.092	46.08%	15.10%	30.59%	8.24%
11	*VcF3′5′H11*	508	56877.28	7.14	0.002	41.73%	14.76%	32.68%	10.83%
12	*VcF3′5′H12*	512	57150.73	8.86	−0.067	45.70%	15.43%	30.86%	8.01%
13	*VcF3′5′H13*	508	57067.51	8.17	−0.04	41.73%	14.76%	33.07%	10.43%
14	*VcF3′5′H14*	508	56992.42	7.67	−0.017	41.93%	14.17%	33.07%	10.83%

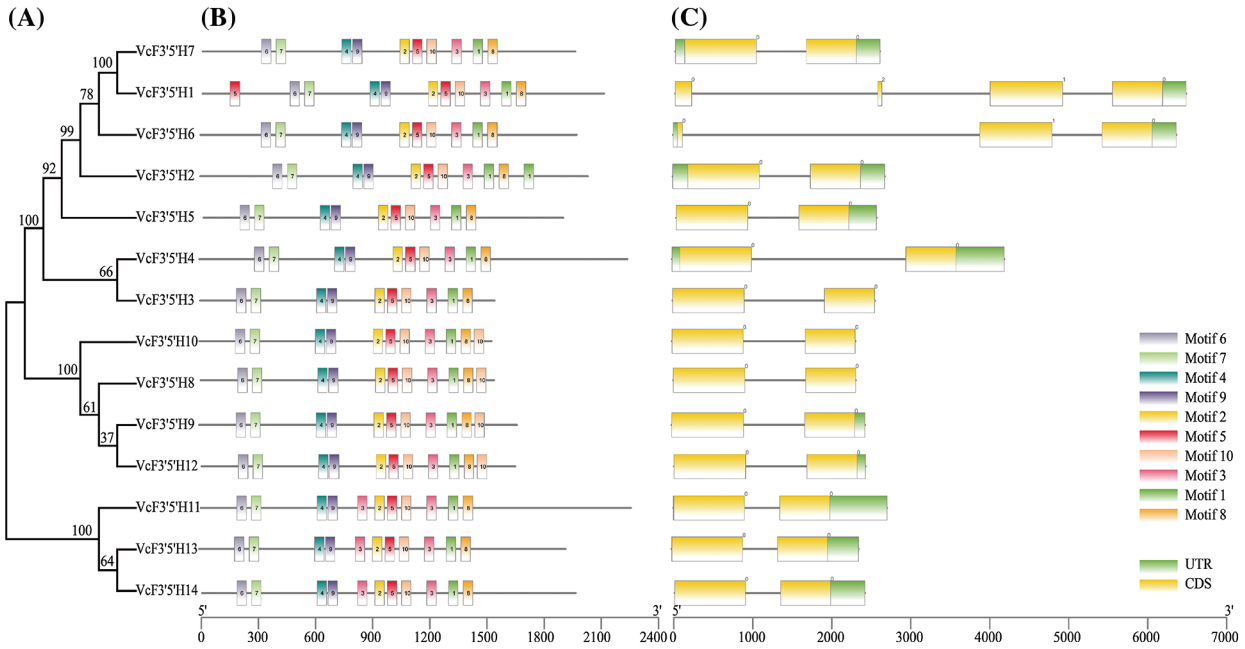

Figure 2: Phylogenetic tree (A), conserved motifs (B), and gene structure analysis (C) of the blueberry *F3′5′H* gene family. A neighbor-joining phylogenetic tree was constructed by aligning the full-length amino acid sequences of the 14 *F3′5′H* genes in blueberries

3.2 Sequence Alignments and Chromosomal Distribution of the VcF3′5′H Genes

As shown in Fig. 3A, the proline-rich region is regarded as a "hinge" required for the optimal orientation of the P450 enzyme. The under-labeled asterisk motif, which formed a binding pocket for oxygen molecules needed for catalytic activities, was present in the blueberry *F3′5′H* genes. The absolutely conserved EXXR motif used to stabilize the core structure, and the most characteristic P450 consensus sequence FxGxRxCxG (heme-binding domain), which was responsible for carbon monoxide-binding ability, were also found in the blueberry *F3′5′H* genes. The LPPGP domain is involved in a variety of biological processes, including protein transport, receptor signal transduction, mRNA splicing, apoptosis regulation, and stress responses to biotic and abiotic stresses. These conserved domains may be associated with the specific functions of the members of this family. The 14 *F3′5′H* genes were mapped to nine scaffolds of the *Vaccinium corymbosum* genome sequence assembly based on their localization information (Fig. 3B). These genes were relatively uniformly distributed. For example, except for scaffold5, scaffold8, and scaffold9, which had two or three genes, there is only one scaffold.

3.3 Analysis of VcF3′5′H cis-Elements

Using the Bedtools software, the 2000 bp upstream of the blueberry *VcF3′5′H* genes was extracted, predicted, and analyzed (Fig. 4). The results showed that *VcF3′5′H* contained numerous basic elements, such as CAAT-box and TATA-box, and various cis-acting elements, including ABRE for abscisic acid responses, ARE for anaerobic responses, CGTCA-motif involved in methyl jasmonate, ACE, G-box and AE-box involved in the light-responsive element, O_2-site involved in the zein metabolic regulatory element, TC-rich repeats involved in the defense and stress response, MBS involved in Drought-induced MYB binding site, as well as AC-I, AP-I, and other motifs. In summary, many cis-acting elements upstream of *VcF3′5′H* were found to be involved in different regulatory processes, indicating that the *VcF3′5′H* gene family has multiple functions.

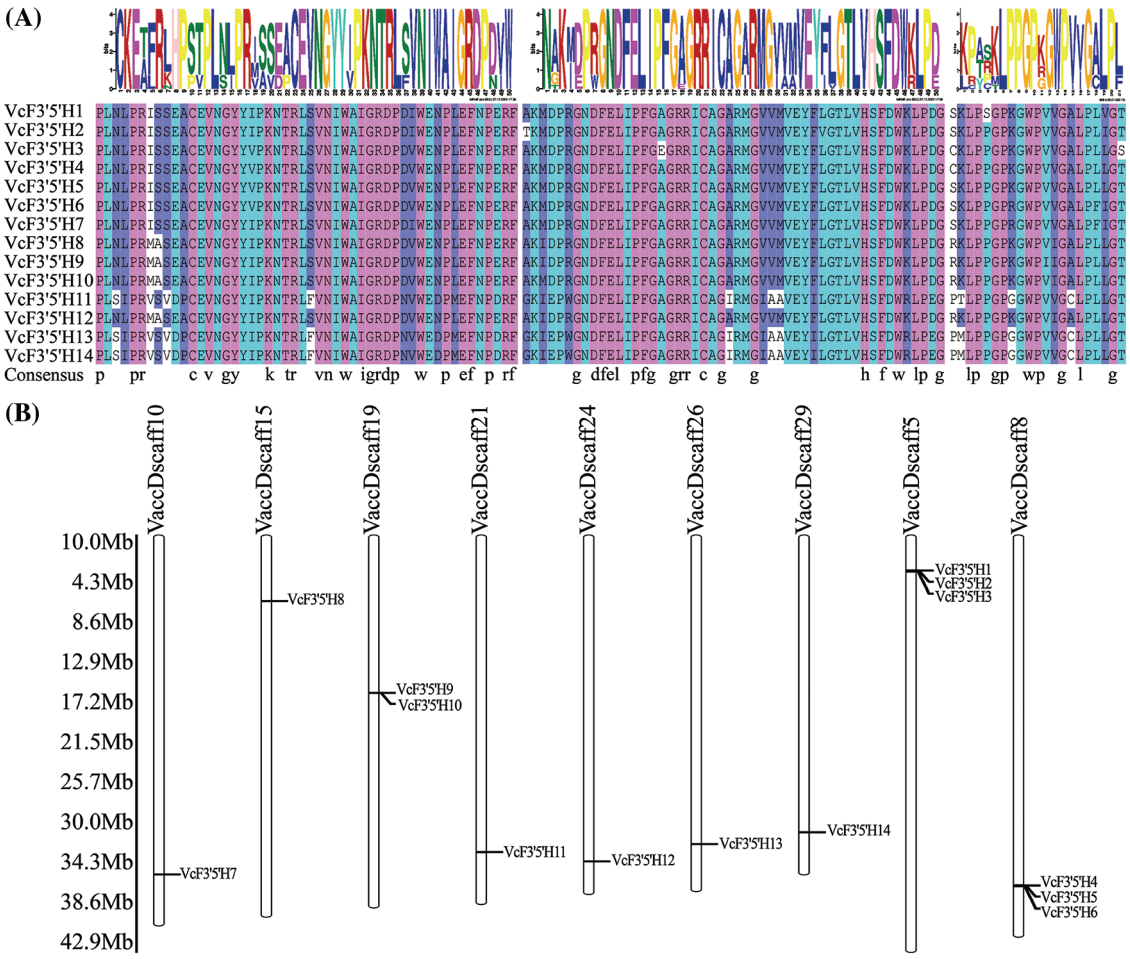

Figure 3: Sequence alignment analysis of *VcF3'5'H* family members (A); chromosomal map of the position of *VcF3'5'H* related genes on chromosomes (B)

Note: Figure A shows the EXXR, FxGxRxCxG, and LPPGP domains from left to right.

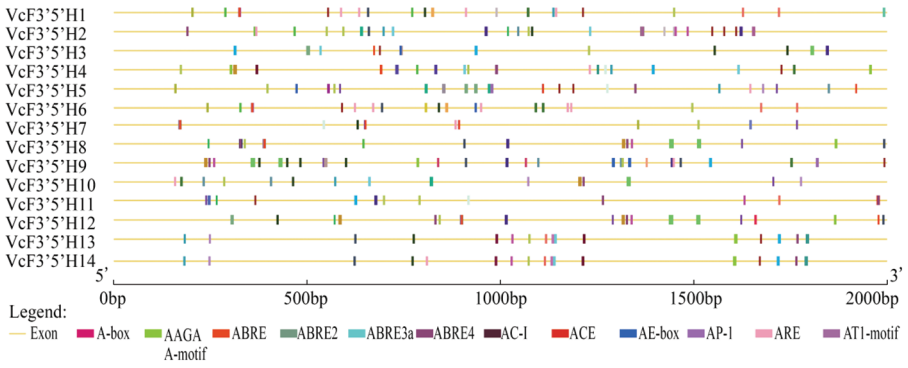

Figure 4: Analysis of the cis-acting elements of *VcF3'5'H* genes

Note: This figure only shows elements with large numbers.

3.4 Identification of VcF3′5′H Genes Regulating Anthocyanins in Blueberries

As shown in Fig. 5, the co-expression network analysis of *F3′5′H* and anthocyanins revealed that *VcF3′5′H3* and *VcF3′5′H4* were both connected to anthocyanins ($R > 0.9$). In particular, *VcF3′5′H4* was related to the synthesis of six different types of anthocyanins, with Petunidin-3-*O*-arabinoside and Delphinidin-3-*O*-arabinoside being the most prominent. According to the data, *VcF3′5′H4* was essential for the synthesis of anthocyanins in blueberries.

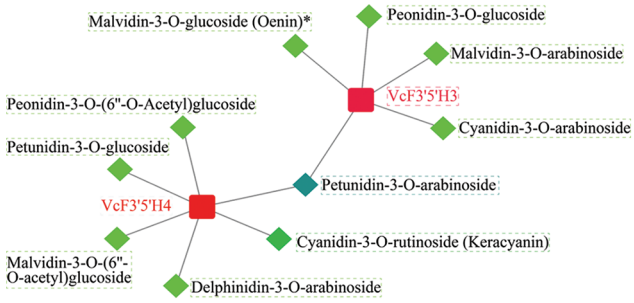

Figure 5: Analysis of the anthocyanin content in blueberries and co-expression analysis of *F3′5′H*. The correlation coefficient of the *F3′5′H* gene and anthocyanin content is 0.90

3.5 qRT-PCR

The *F3′5′H* gene family is related to fruit coloration. To explore the relationship between the *F3′5′H* gene family and the fruit coloration of blueberries, we detected the expression levels of the *F3′5′H* gene family in three stages using qRT-PCR. The results showed that except *VcF3′5′H8*, *VcF3′5′H10*, *VcF3′5′H11*, *VcF3′5′H12*, and *VcF3′5′H13*, the genes were highly expressed at ripening stage (Fig. 6). In particular, *VcF3′5′H4* might play a key role in the formation of fruit color. These results indicate that the *F3′5′H* gene family is indeed involved in the color formation of purple fruit.

3.6 Cloning and Subcellular Localization of the VcF3′5′H4 Gene

The plant expression vector of *VcF3′5′H4*, pBWA(V)HS-*VcF3′5′H4*-GFP, was constructed and injected in *N. benthamiana* leaves. The fluorescence expression was observed under a laser confocal microscope (Fig. 7). Combining the observation results with a bioinformatics predictor (Cell-PLoc 2.0), we speculated that *VcF3′5′H4* was located in the endoplasmic reticulum.

4 Discussion

4.1 Analysis of the Basic Characteristics of Blueberry F3′5′H

Cytochrome P450 protein family contains 10 subfamilies [44], and *F3′5′H* belongs to the CYP75A subfamily [33,45]. The members of the other nine families are mainly involved in the metabolism of sterols, fatty acids and hormones, while the members of the *CYP75A* subfamily are involved in the synthesis of secondary compounds, such as phenylpropanes, flavonoids, isoflavones, and alkaloids. For example, the overexpression of *F3′5′H* genes can promote the accumulation of poplar anthocyanins and enhance the hydroxylation of flavonoids [22]. The hydroxylation pattern of flavonoids is largely determined by *F3′5′H* genes. The cloning and characterization of *F3′5′H* genes are of great significance. For example, cloning *F3′5′H* genes can change the color of plants, such as roses and carnations that do not naturally have *F3′5′H* activities, from lavender to blue [23–25].

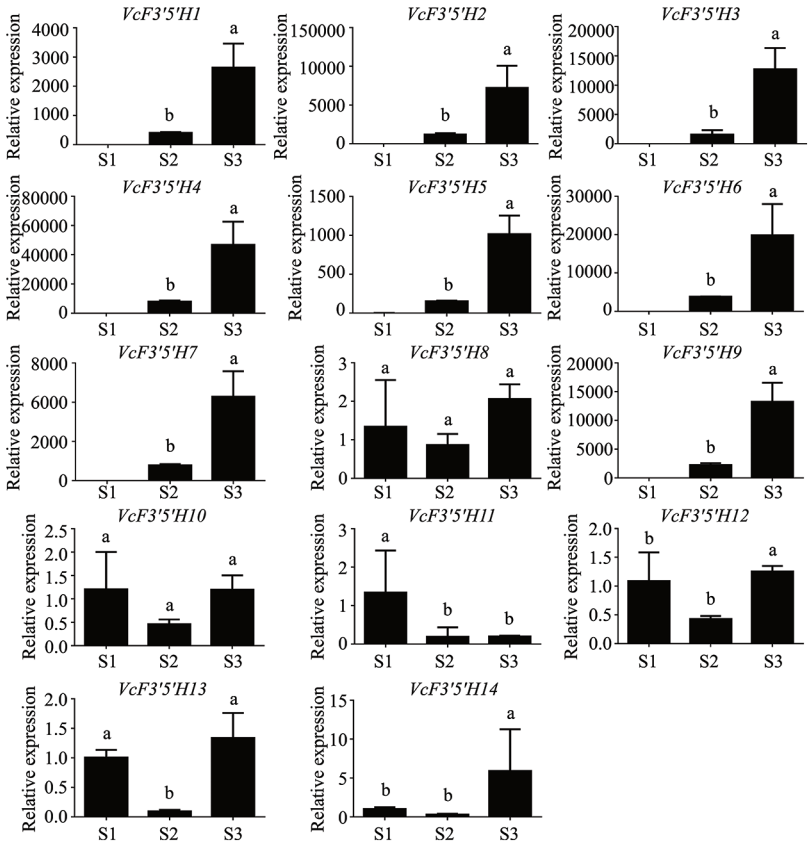

Figure 6: Expression of *F3′5′H* genes at different developmental stages

Some studies focused on *F3′5′H* genes in plants [20,21,46]. In these studies, blueberry genes were identified with bioinformatics analyses. It was found that these proteins were basically hydrophilic proteins with 508–601 amino acids. The members were relatively conserved and had flavonoid hydroxyl enzyme-specific conserved domains, such as proline-rich "hinge" regions, EXXR motif heme-binding domains, and substrate recognition sites [30,47,48]. In particular, the LPPGP domain was involved in various biological processes, including protein trafficking, receptor signal transduction, mRNA splicing, apoptosis regulation, and stress responses to biotic and abiotic stresses [49]. This result is consistent with that of previous studies on tobacco, petunia, moss, and tea trees [33,35,50]. For example, a study showed that most genes from the same gene family have similar numbers of exons and introns but very different lengths of introns [51]; our experiment showed that the genes in the *VcF3′5′H* gene family mostly have two exons, with some genes having three or four. In addition, some research has demonstrated that F3′5′H proteins are mainly located in the endoplasmic reticulum membrane and are involved in the metabolic pathways of fatty acids, sterols, hormones, and secondary metabolites [33]; the results of our study are consistent with those of previous research, and the conclusion can be drawn that the structural genes of the anthocyanin synthesis pathway generally function on the endoplasmic reticulum [52]. It has also been reported that the amino acid sequences of F3′5′H are diverse, but their folding and structures remain highly conserved throughout evolution. The results of our study are in agreement with those of previous studies [53,54].

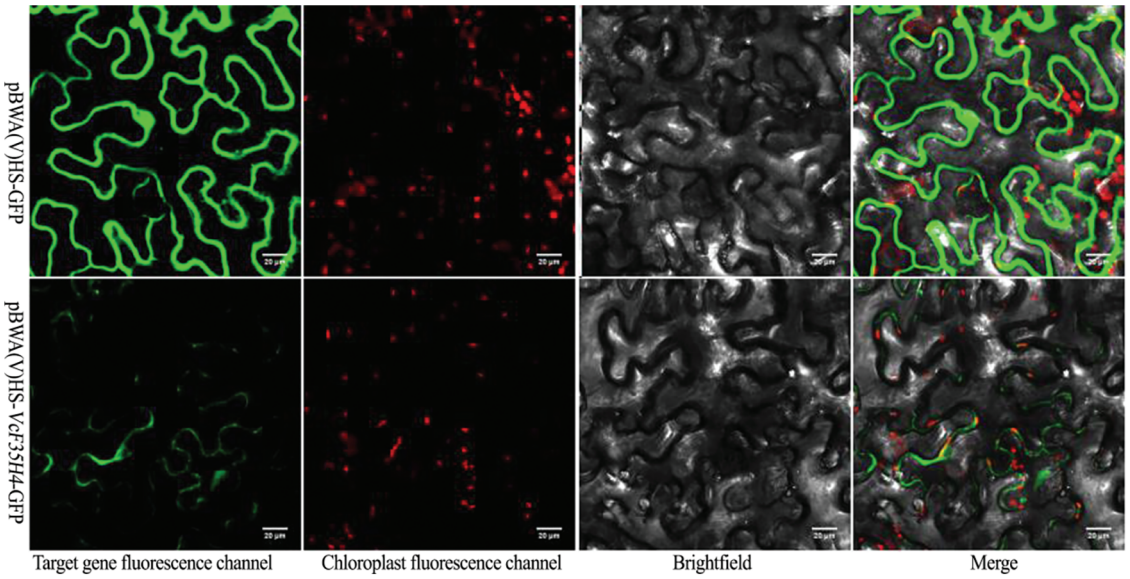

Figure 7: Map of VcF3′5′H4 protein subcellular locations
Note: pBWA(V)HS-VcF3′5′H4-GFP fusion protein and pBWA(v)HS-GFP after the injection of *N. benthamiana* for 48 h were observed under a laser confocal microscope, and pBWA(v)HS-GFP was used as a control.

4.2 Functional Analysis of Blueberry F3′5′H

Numerous studies have shown that *F3′5′H* genes are involved in the synthesis of anthocyanins [22,55–57]. The synthesis pathway of anthocyanins needs to be induced by light, and light-inducible genes often contain cis-acting elements, such as GT-1 motif and G-box. Some researchers believed that when the light time prolonged, the expression levels of *PAP1* (MYB) and *TT8* (bHLH) in *Arabidopsis thaliana* seedlings were increased, improving the expression levels of key synthesis enzyme genes for anthocyanins, such as CHS, F3H, and DFR. The probable reason is that these candidate genes have three cis-acting elements, including MRE (light-responsive MYB binding site), ACE, and G-box, which can regulate gene expression levels [58–60]. This also indicates that light-responsive elements, such as GT-1 motif, G-box, and ACE, can regulate the synthesis of anthocyanins through light intensity. Wang et al. [61] found that the *CsF3′5′H* gene promoter of the tea plants contained multiple light-responsive elements by cloning it. The results in our study are consistent with the findings in the previous research, indicating that this promoter may synthesize anthocyanins mediated by light.

Sun et al. [57] confirmed that the heterologous expression of PCFH belonging to the CYP75B subfamily could produce a kind of delphinidin derivative (3′,5′-hydroxylated anthocyanidins) *in vivo* and its encoded protein had the 3′,5′-hydroxylation function. Takatori et al. [62] found that *F3′5′H* genes were involved in the synthesis of delphinium in *Eustoma grandiflorum* (Raf.) Shinn, and of other types of anthocyanins, such as cyanidin-type anthocyanin and pelargonidin-type anthocyanin that produce various flower colors. In our study, the co-expression network analysis of *F3′5′H* and anthocyanins revealed that *VcF3′5′H3* and *VcF3′5′H4* were both connected to anthocyanins ($R > 0.9$). In particular, *VcF3′5′H4* was related to the synthesis of six different types of anthocyanins, with Petunidin-3-*O*-arabinoside and Delphinidin-3-*O*-arabinoside being the most prominent. In line with the results in previous studies, our findings indicate that the *VcF3′5′H4* genes mainly induce the synthesis of delphinium in blueberries.

5 Conclusions

In this research, we identified 14 *VcF3′5′H* genes. The *VcF3′5′H* gene and anthocyanin content co-expression confirmed that the *VcF3′5′H* family was closely related to the synthesis of anthocyanins. In particular, *VcF3′5′H4* played a key role. Based on the prediction using Cell-PLoc 2.0, we hypothesized that *VcF3′5′H4* was localized in the endoplasmic reticulum. This study lays a theoretical foundation for further research on the function of the *F3′5′H* gene family in blueberries.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

Table S1: 14 *VcF3′5′H* genes co-expressed with anthocyanin

Table S2: Quantitative primer sequences

Table S3: *VcF3′5′H4* primer sequence

Figure S1: 17 *VcF3′5′H* genes and P450 subfamily sequences for Phylogenetic tree