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Cloning and Analysis of *RrF3'H* in *Rosa rugosa*

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Abstract

Rosa rugosa is an important garden ornamental plant which belongs to the genus *Rosa* of the family *Rosaceae*. The current wild and cultivated *R. rugosa* are mostly purple, pink, a small amount of white, but lack of yellow, orange and so on. Flavonoids 3'-hydroxylase belongs to CYP75B subfamily of cytochrome P450, and is an essential enzyme in anthocyanins synthesis. In this experiment, *RrF3'H* gene was cloned from the petal of *Rosa rugosa* 'Hunchun' using RT-PCR, and bioinformatics analysis was performed. The *RrF3'H* gene's full length of opening reading frame was 1687 bp, encoding 510 amino acids. The formulas of proteins encoded by *RrF3'H* were $C_{2666}H_{4149}N_{699}O_{734}S_{24}$. The derived protein had a molecular weight of 58,506.95 Da. The aliphatic index was 90.94. It belongs to unstable hydrophilic protein. The protein consists of 46.76% α-helix, 31.04% random coil, 7.66% β-corner and 14.54% extended strand. The protein contains 21 Ser phosphorylation sites, 12 Thr phosphorylation sites, and 2 Tyr phosphorylation sites. The protein contained two O-glycosylation sites, located at positions 98 and 263 of the amino acid sequence respectively. The protein has a signal peptide site and a transmembrane structure. In addition, by comparing the expression levels of *RrF3'H*, we found *RrF3'H* was positively correlated with the depth of flower color.

Keywords

Rosa rugosa, *F3'H*, Bioinformatics Analysis, Gene Expression

1. Introduction

Rosa rugosa originated in China, and it belongs to the genus *Rosa* in the family *Rosaceae*. As an important ornamental garden plant, it has graceful shape, sweet-smelling flowers and many varieties. However, the petal colors of *R. rugosa* are mostly pink, purple and white, but lack of other colors [1] [2] [3]. This

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monotone coloration seriously limits the use of *Rosa rugosa* in gardens.

Flavonoid 3'-hydroxylase belongs to the CYP75B subfamily of the P450 family and is essential for the synthesis of anthocyanins. It can catalyze the formation of dihydroquercetin from dihydrokaempferol [4] [5]. Brugliera *et al.* (1999) [6] cloned the first *F3'H* gene from *Petunia hybrida*, and it has been cloned from various plants so far, such as *Dendranthema morifolium*, *Ginkgo biloba*, *Vitis amurensis*, *Chimonanthus praecox* and *Phalaenopsis aphrodite* [7] [8] [9] [10]. Yang yuxia *et al.* (2013) [10] found that the expression level of *F3'H* in *Phalaenopsis aphrodite* with red flowers was about 19 times that of *Phalaenopsis aphrodite* with yellow flower, indicating that *F3'H* had an important influence on the synthesis of anthocyanin. *Petunias* with red flowers [11] were obtained by reducing the expression of *F3'H* gene and over expression of *DFR*. By inhibiting the expression level of *F3'H* gene in corydalis viola, it was also found that the content of anthocyanin was significantly reduced and the flower color became lighter [12]. All these studies indicate that the study of *F3'H* gene is of great significance to the improvement of plant color.

In this study, *F3'H* gene was cloned from petals of *R. rugosa*, and analysed its bioinformatics and gene expression in different flowering stages, in order to lay a foundation for future research on pigment formation and color improvement in *R. rugosa*.

2 Materials and Methods

2.1. Plant Materials

Petals were collected from *R. rugosa* 'Hunchun', *R.* 'Jiaomeisanbian', *R.* 'Miaoyu' (**Figure 1**). From mid-April to the beginning of May 2016, we collected the petals at the soft bud stage, initial opening stage, full opening stage and wilting stage at Shandong Agricultural University Rose Planting Garden, Tai'an City (36˚18'N, 117˚13'E), Shandong Province, China. After picking the petals, we placed them in liquid nitrogen and then stored them at −80˚C for further use.

2.2. Methods

2.2.1. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from petals of different stages using an EASY Spin

Figure 1. Flowers used in this experiment. (a) *Rosa rugosa* 'Hunchun'. (b) *Rosa rugosa* 'Jiaomeisanbian'. (c) *Rosa rugosa* 'Miaoyu'.

Plant RNA Extraction Kit (Aidlab Biotechnologies Co., Ltd.); then, the RNA concentration, purity and integrity were determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA) and 1.0% nonvariable agarose gel electrophoresis. First-strand cDNA was synthesized directly from the tested RNA samples, and the reaction was performed according to the method of the 5X All-in-One RT MasterMix reverse transcription kit (abm Inc., USA).

2.2.2. Cloning of *F3'H*

'Hunchun' cDNA was used as the template. The specific primers were designed using Primer 5 software and based on the gene fragment in the *R. rugosa* transcriptome sequencing results (Table 1). Specific amplification of the *F3'H* open reading frame (ORF) was carried out with reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) using cDNA as the template. PCR reaction system was as follows: ddH_2O 9.5 μl, 2 × EasyTaqSuperMix 12.5 μl, target gene upstream primer 1 μl and downstream primer 1 μl, template cDNA 1 μl, 25 μl in total. Reaction conditions as follows:: 94˚C for 5 min; 94˚C for 30 s, 53˚C for 30 s, and 72˚C for 1 min, 35 cycles; and then extension at 72˚C for 10 min. PCR products were detected by 1% agarose gel electrophoresis. According to the instructions of Hipure Gel Pure DNA Mini Kit (Magen), the target strip was recovered, then connected with the pmd18-t vector of TaKaRa, transformed into *E. coli* DH5a, identified by PCR, then the positive clone was selected for sequencing.

2.2.3. Bioinformatics Analysis of *F3'H*

Online Blast provided by NCBI was used for alignment of homologous sequences. DNAMAN was used to compare the protein with other plant proteins. The basic physicochemical properties of *RrF3'H* were predicted by ProtParam in ExPasy server. The NCBI CD-search function was used to predict the conserved domain of target genes. ORF Finder was used to search the open reading frame of *RrF3'H* gene cDNA. ProtScale was used to predict the hydrophobicity of proteins. Online software NetPhos 3.1 server, NetOGlyc 4.0 server and SignalP 4.1 were used to predict the phosphorylation site, glycosylation site and signal peptide of the target gene coding protein. The transmembrane domain of RrF3'H protein was predicted by TMHMM. SOPMA was used to predict the secondary structure of the protein encoded by *RrF3'H*. MEGA 5.0 was used to construct the *RrF3'H* phylogenetic tree.

Table 1. Primers used to clone and expression analysis of *RrF3'H*.

Primer name	Nucleotide sequence (5'-3')	Purpose
RrF3'H-F	ATGGAGGCTTCAGTTTCTTGG	Intermediate fragment amplification
RrF3'H-R	AGATGGATTGGAAGCCGAG	
RrF3'H-1	GGATGGAGGAAGCTTGTGG	
RrF3'H-2	CTCGGCTTCCAATCCATCT	3' RACE amplification
B26	GACTCTAGACGACATCGATTTTTTTTTTTTTTTT	

2.2.4. Gene Expression Analysis

Total RNA extraction and cDNA synthesis were referenced to Section 2.2.1. The expression levels of *RrF3'H* gene in 4 different flowering stages (soft bud stage, initial opening stage, full opening stage and wilting stage) from *R. rugosa* 'Hunchun', *R.* 'Jiaomeisanbian', *R.* 'Miaoyu' were analyzed via qRT-PCR on a Bio-Rad CFX96TM Real-Time PCR instrument (Bio-Rad, Inc., USA). The qRT-PCR mixture (20 μL total volume) contained 10 μL of SYBR® Premix Ex Taq™ (TaKaRa, Inc., Japan), 7.2 μL of ddH_2O, 0.4 μL of each primer and 2 μL of cDNA. The PCR program was carried out with an initial step of 95˚C for 30 s; 40 cycles of 95˚C for 5 s and 60˚C for 30 s; and then 95˚C for 10 s, 65˚C for 5 s and 95˚C for 5 s for the dissociation stage. Each gene was assessed with three biological replicates. The relative expression levels of the genes were calculated via the $2^{-\Delta\Delta Ct}$ method [13].

3. Results and Analysis

3.1. Cloning and Sequence Analysis of *RrF3'H* Gene

The *RrF3'H* intermediate fragment of 1523 bp was obtained by amplification and sequencing (Figure 2), and the 3'-terminal sequence of 183 bp was obtained after 3' RACE amplification (Figure 2). The full length of the cDNA sequence of 1687 bp was obtained by splicing the two fragments using DNAstar. DNAMAN was used to analyze the base sequence of *RrF3'H*, and it was found that *RrF3'H* included a complete open reading frame (ORF) containing the starting codon ATG and the ending codon TAA, a complete reading frame (ORF) with a length of 1530 bp, encoding 510 amino acids (Figure 3).

DNAMAN software was used to compare the multiple sequences of *F3'H* protein amino acids in 7 plants, including *Rosa rugosa*. According to the comparison results (Figure 4), *F3'H* was highly conserved in different plants, indicating that *F3'H* homology of different species was very high.

Using MEGA5 to build system phylogenetic tree of the amino acid sequence of 16 kinds of plants (Figure 5), including *R. rugosa*, it can be seen that *R. rugosa* was closely related to the members belonging to *Rosaceae* family. The *R. rugosa* and *Prunus persica* converged first means *R. rugosa* has the most close relationship

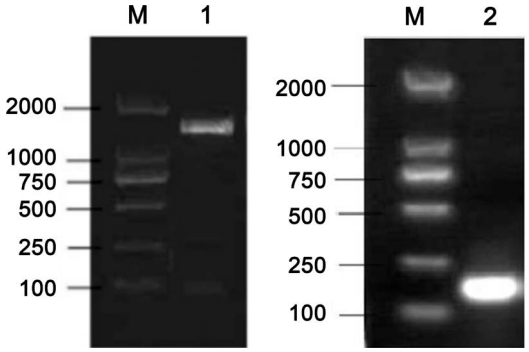

Figure 2. PCR amplification of *RrF3'H*. M: Marker; 1: Intermediate fragment; 2: 3'-terminal fragment.

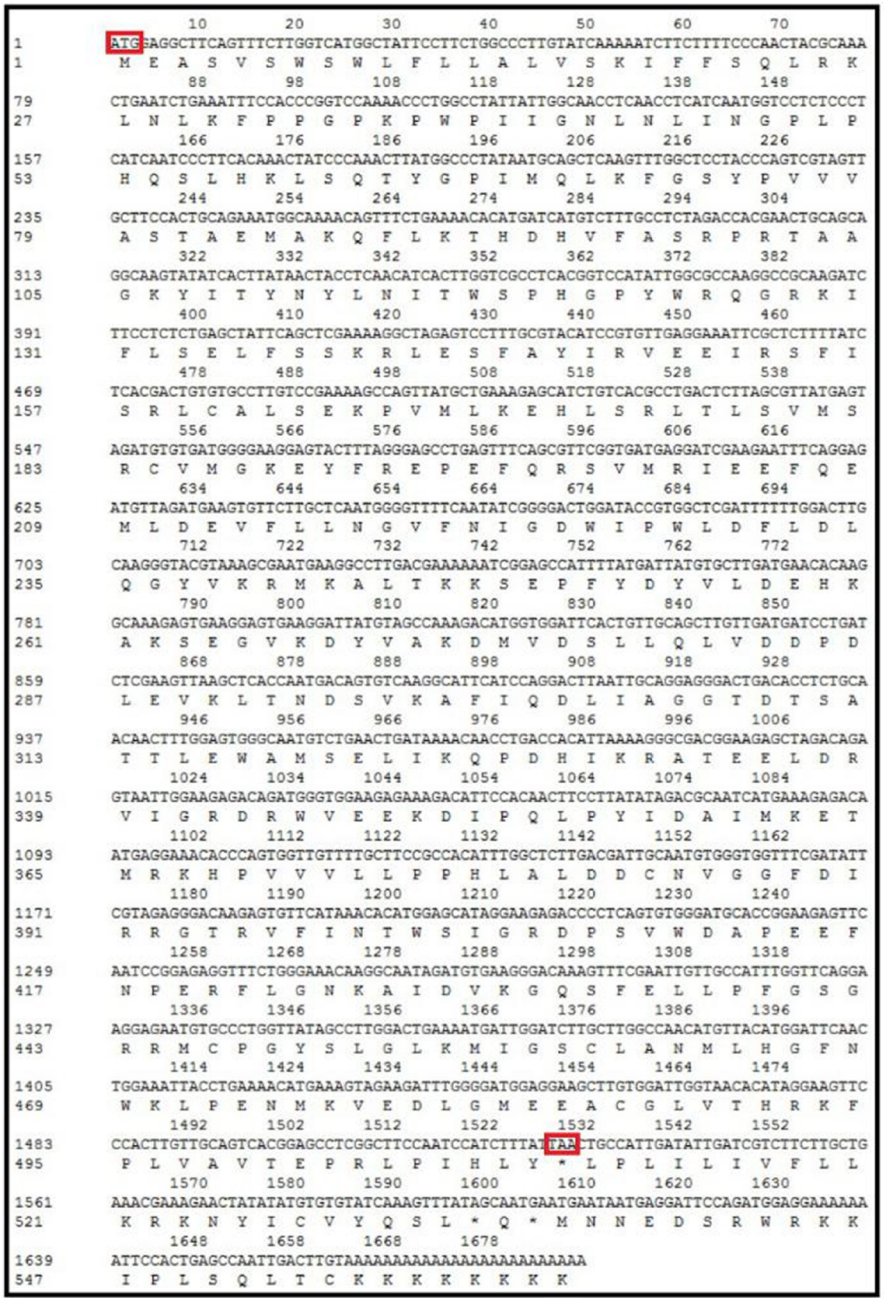

Figure 3. *RrF3'H* cDNA nucleotide sequence and the amino acid sequence.

with *Prunus persica*. Then converged with *Prunus mume* and *Narcissus tazetta* means that *R. rugosa* also has a very close relationship with *P. mume* and *N. tazetta*. In addition, they converged to a large group with *Prunus cerasifera* and *Paeonia lactiflora*, but it was relatively distant from other plants.

3.2. Bioinformatics Analysis

RrF3'H belongs to the P450 superfamily, with corresponding conservative structure domains. The formulas of proteins encoded by *RrF3'H* were $C_{2666}H_{4149}N_{699}O_{734}S_{24}$. The derived protein had a molecular weight of 58506.95 Da, a calculated pI of

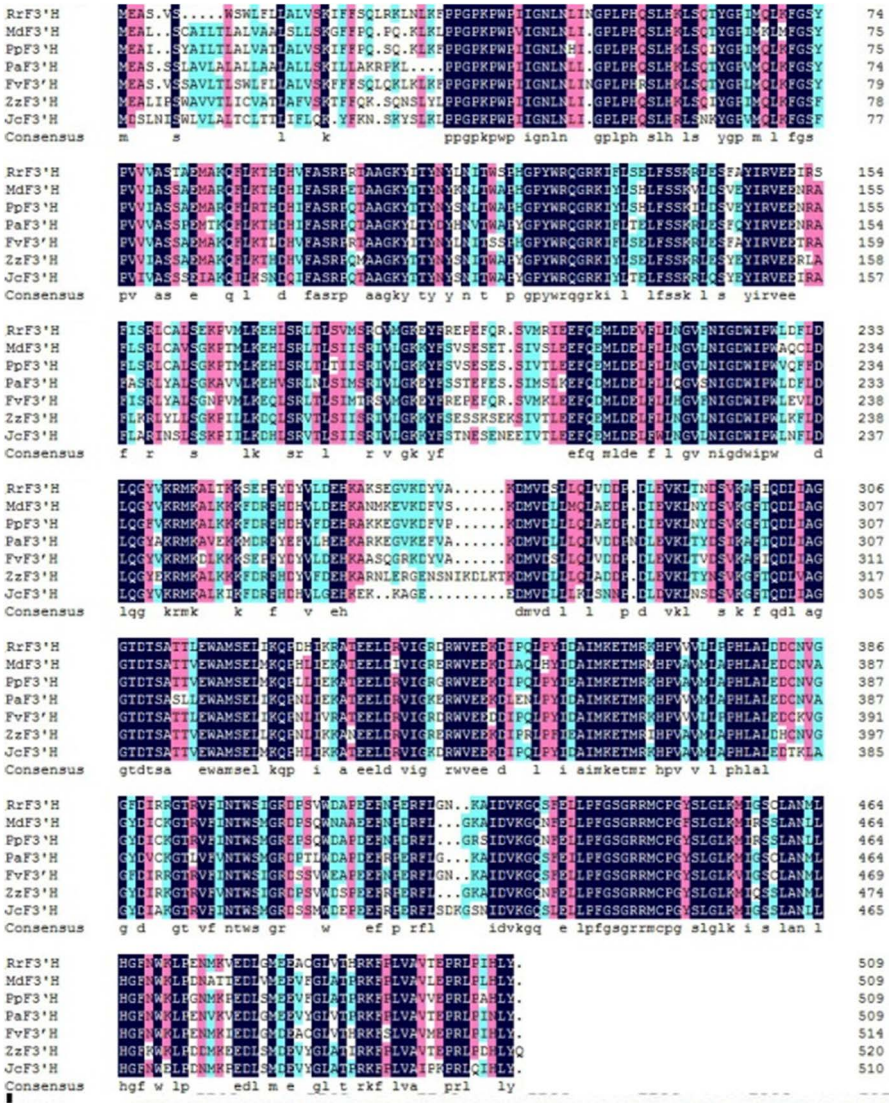

Figure 4. Multiple alignment of *F3'H* sequences of different plants.

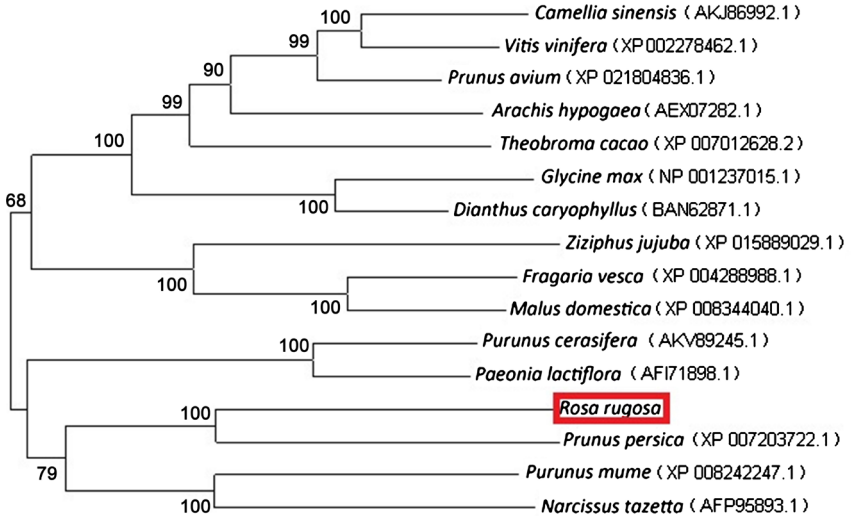

Figure 5. The phylogenetic tree of the amino acid sequences of *F3'H*.

7.65. Instability index of the protein is 45.18 (>40), so it can be speculated that *RrF3'H* encoded protein is unstable protein. The aliphatic index was 90.94. The grand average of hydropathicity is −0.207, which means it's hydrophilic protein. The secondary structure prediction result demonstrated that the protein consists of 46.76% α-helix, 31.04% random coil, 7.66% β-corner and 14.54% extended strand. The protein contains 21 Ser phosphorylation sites, 12 Thr phosphorylation sites, and 2 Tyr phosphorylation sites. The protein contained two O-glycosylation sites, located at positions 98 and 263 of the amino acid sequence respectively. In addition, the protein has a signal peptide site and a transmembrane structure.

3.3. Expression Patterns of *RrF3'H* in Different Flowering Stages

The expression levels of *RrF3'H* were compared among the petals of *R.* 'Hunchun', *R.* 'Jiaomeisanbian', *R.* 'Miaoyu' at different stages (soft bud stage, initial opening stage, full opening stage and wilting stage). The expression patterns of the gene are shown in Figure 6. The results showed the expression level of this gene was the highest in the full openning stage, and the lowest in the soft bud stage. It shows a trend of rising first and then falling. In addition, in each opening stage, the expression level of *RrF3'H* was the highest in 'Hunchun', followed by 'Jiaomeisanbian', and the expression level was the lowest in 'Miaoyu'.

4. Discussion

Previous research has clearly shown that flower color intensity is largely determined by the amount of accumulated anthocyanins, and the anthocyanin biosynthetic pathway is well known [14] [15] [16]. Flavonoid 3'-hydroxylase (*F3'H*) is one of the key enzymes in the synthesis of anthocyanins in plants. It can catalyze the formation of dihydroquercetin from dihydrokaempferol, therefore, it plays an important role in the formation of plant color [5] [17]. In addition, in *R. rugosa*, *F3'H* competes with *DFR* and *FLS* for the common substrate dihydrokaempferol, so *F3'H* is of great significance for changing the color of *R. rugosa*. In this study, the *RrF3'H* gene was successfully cloned from *R. rugosa*, and analysed

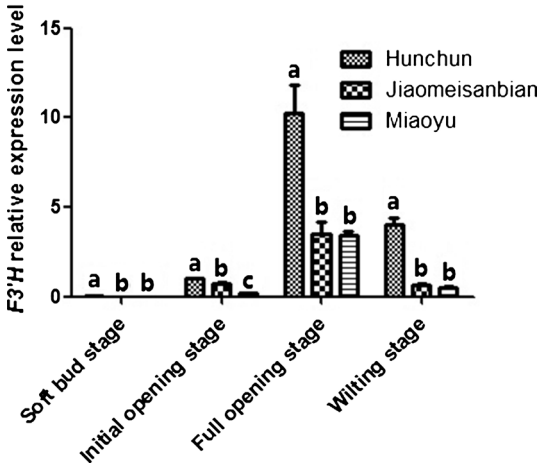

Figure 6. Relative expression levels of *RrF3'H*.

its bioinformatics. *RrF3'H* gene contains 1687bp open reading frame, encodes 510 amino acids, its molecular formula is $C_{2666}H_{4149}N_{699}O_{734}S_{24}$, its molecular weight is 58,506.95 Da, and these features are similar to those found in most plants [18]. By comparing the amino acid sequences of *RrF3'H* and the corresponding proteins in other plants, we found that the amino acid sequences of *RrF3'H* have higher homology with those of other plants, indicating that *F3'H* is relatively conservative among different species. The secondary structures were composed of α-helix, random coil, β-corner and extended strand. The α-helix domain can cause the bilayer of phospholipids to bend inward, which can resist the cell membrane damage caused by low temperature and protect the cell structure [19]. *RrF3'H* has multiple phosphorylation sites, indicating that reversible phosphorylation regulation plays an important role in achieving its functions.

By comparing the expression levels of *RrF3'H* among the petals at different stages, we found the expression level of this gene was the highest in the full openning stage, and the lowest in the soft bud stage. It shows a trend of rising first and then falling. Therefore, the full opening stage may be the time when anthocyanins are synthesized in large quantities in *R. rugosa*. By comparing the expression level of *RrF3'H* gene in the petals of the three varieties, we found that the expression level of *RrF3'H* was positively correlated with the depth of flower color; the gene expression level will be higher in redder flowers. It showed the highest expression level in 'Hunchun' and the lowest expression level in 'Miaoyu'. Therefore, *RrF3'H* is indeed related to the formation of flower color in *R. rugosa*, and the higher the expression of *RrF3'H* gene, the more the anthocyanin synthesis. In this study, *F3'H* gene in *R. rugosa* petals was isolated and analyzed to find out the information of this gene, which provided a theoretical basis for the improvement of *R. Rugosa*'s color in the future.

5. Conclusion

We successfully cloned the *RrF3'H* from the *R. rugosa*, and the protein encoded by this gene is highly similar to that in other plants. In addition, this gene plays an important role in the formation of the color of *R. rugosa* and is positively correlated with the amount of anthocyanin synthesis.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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