Cloning and Expression Analysis of \textit{RrRUP2} Gene Related to Photomorphogenesis Biosynthesis in \textit{Rosa rugosa}

Yenan Wang*, Mingyuan Zhao*, Xu Han, Lanyong Zhao*, Zongda Xu#

College of Forestry, Shandong Agricultural University, Taian, China
Email: *sdzly369@163.com, *xuzoda@163.com

Abstract

Plants have evolved and perfected a series of light receptors to feel the light at different bands and regulate the expression, modification and interaction of related genes in plants through signal transduction. So far, many photoreceptors have been identified in plants, \textit{UVR8} has recently been identified as a receptor for \textit{UV-B} light. This paper cloned a WD40 gene related to \textit{UVR8} protein subunit, named \textit{RrRUP2}, based on the \textit{Rosa rugosa} transcriptome data, using \textit{Rosa rugosa} “Zi zhi” as experimental materials. The full length of cDNA of the gene was obtained by RT-PCR and RACE methods. The total length of this gene is 1173 bp, and it encodes 390 amino acids. After bioinformatics analysis, the molecular formula \textit{C}_{3415}\textit{H}_{5659}\textit{N}_{1173}\textit{O}_{1434}\textit{S}_{313} was predicted; the relative molecular weight was 96129.27 Da; the theoretical isoelectric point PI value was 5.00; and its instability index was 47.06. The total average hydrophobic index was 0.750. In the secondary structure of \textit{RrRUP2} protein, there are 10 \textit{\alpha}-helix, 45 \textit{\beta}-helix, 181 Random coil, and 154 Extended strand. Gene Bank Blast results showed that the amino acid sequence encoded by \textit{RrRUP2} was more than 90% homologous with the \textit{RUP2} protein of \textit{Rosa chinensis}, Fragaria, Malus, Pyrus, Juglans, Arabidopsis and Tobacco, so it can be inferred that the \textit{RrRUP2} gene is a WD repeat-containing protein. Regarding to fluorescence quantitative expression analysis of \textit{RrRUP2}, we find its expression pattern is corresponded with the accumulation of anthocyanins.

Keywords

\textit{Rosa rugosa}, UV-B, \textit{UVR8}, \textit{RUP2}, Photomorphogenesis, Anthocyanin, Gene Expression

*These authors contribute equally. * Corresponding authors.
1. Introduction

*Rosa rugosa* is an ornamental shrub of the *Rosaceae* family, because of its beautiful pattern, unique fragrance and color; people call it “love flowers” and “gold flowers” [1]. There are many varieties of roses, but most roses are darker in color; how to improve rose color is particularly important. The color changes of plant petals are mainly related to the synthesis and regulation of anthocyanin, the biosynthesis and regulation of anthocyanin is accomplished by the complex network of the genetic background and environmental factors [2] [3]. The coding genes include 15 structural genes and 3 regulatory gene families [4], among which 3 regulatory gene families are MYB, BHLH and WD40. Among them, WD40 protein provides the necessary platform for MBW transcription complex [5]. Coding genes determine the type and time of anthocyanin synthesis, while environmental factors such as light affect the time of anthocyanin biosynthesis [6]. Roses are masculine plant, which like growing with abundant of sunlight environment.

Light is very important for the growth and development of plants, not only providing energy for the whole life activities of plants, but also playing the role of environmental signal factors influencing the whole life cycle of plants from seed germination to flowering and fruting. Therefore, plants have evolved and perfected a series of light receptors to feel the light at different bands and regulate the expression, modification and interaction of related genes in plants through signal transduction. So far, many photoreceptors have been identified in plants, such as photosensitive pigments that sense far-red and red light, cryptochrome that senses blue and UV-A, phototrophic proteins and ZTL, and *UVR8* that has recently been identified as a receptor for UV-B light [7] [8] [9] [10] [11].

UV-B is the b band of ultraviolet, which has a short wavelength and high energy [12]. UV-B radiation affects the synthesis of plant secondary metabolites and increases the content of phenolic compounds, terpenoid and anthocyanins in plant leaves. When plants irradiate UV-B, *UVR8* can be a monomer to transmit light signals; When UV-B is removed, *UVR8* can return to the ground state through dimerisation. Therefore, *UVR8*, as the light receptor that senses UV-B signal, is of great significance for plants to inactivate and return to the ground state. In order to prevent the over-amplification and output of UV-B signal, there is a very accurate negative feedback regulation mechanism in plants, among which RUP2 is a particularly important negative regulator [13].

Some studies have shown that the signal transduction pathway of *UVR8* in UV-B, at present, *UVR8* has been cloned and analyzed in many plants such as *Arabidopsis* [14], *Malus domestica* [15], *Prunus avium* [16] and so on. However, studies on the negative regulatory factor RUP2 in this pathway are absent. In this study, based on the *R. rugosa* transcriptome data, we cloned and identified *RrRUP2* gene from the petals of *Rosa rugosa* “Zi zhi” for the first time. We carried out detailed bioinformatics analysis, homology analysis and the temporal and spatial expression pattern analysis of the *RrRUP2* gene in order to provide...
some useful informations for analyze the mechanism of the regulatory factors in UV-B signaling pathway.

2. Material and Methods

The experiment was conducted from April 2017 to January 2018 in the flower germplasm resources nursery of shandong agricultural university and the flower research institute of forestry university.

2.1. Plant Material

The plant materials, Chinese representative Rosa rugosa “Zi zhi”, Rosa rugosa “Fen zi zhi”, Rosa rugosa “Bai Zi zhi”. R. rugosa “Zi zhi” is purple, R. rugosa “Fen zi zhi” is pink, R. rugosa “Bai zi zhi” is white. Plants were selected from April to May 2017 with robust, stable and pure designs, and the half-open petals of the above three varieties were collected as the experimental materials for gene cloning. The flower petals of the above three varieties were collected in the bud stage, the initial stage, the half stage, the blooming stage and the end of the blooming period as the differential expression test among the varieties; Five flowering stages and root, stem, leaf, pistil, stamen, sepals were collected for tissue differential expression test. All samples were collected directly frozen with liquid nitrogen, and finally stored at −80℃ until used.

2.2. Methods

2.2.1. Total RNA Extraction and cDNA Synthesis

According to the instructions of EASY spin plant RNA rapid extraction kit (Aidlab Biotech, Beijing, China), RNA of all tissue parts and total RNA of R. rugosa “Zi zhi” petals were extracted. Their concentration and purity were determined by uv spectrophotometer. Meanwhile, their integrity was detected by 1% agarose gel electrophoresis (Thermo Fisher Scientific, Wilmington, Delaware, USA). The first strand of reverse transcription synthesis cDNA was synthesized by RNA reverse transcription according to the instructions of abm reverse transcription kit (ABM Company, Vancouver, Canada).

2.2.2. PCR Cloning of Anthocyanin Biosynthesis Related Gene

According to the notes of the R. rugosa-transcriptome database, 48 Wd40 genes were isolated and specific primers were designed using Oligo7.0 software (Table 1). The reaction system included 1 µL cDNA, 1 µL F1 primer (10 µmol/L), 1 µL R1 primer (10 µmol/L), and 12.5 µL PCR MIX, with ddH2O added to a total volume of 25 µL. The reaction conditions were: 94℃ for 5 min; 94℃ for 30 s, 53℃ for 30 s, and 72℃ for 1 min for a total of 35 cycles; and then extension at 72℃ for 10 min. Next, 1% agarose gel electrophoresis was used to detect the PCR products. The target PCR fragment was recovered with the Hipure Gel Pure DNA Mini Kit (Magen). The recovered fragment was ligated to the pMD18-T vector and then transformed into Ecoli DH5α. The positive clones were selected and sent to BGI for sequencing.
Table 1. Primers used in the present study.

<table>
<thead>
<tr>
<th>Primer name (5′ 3′)</th>
<th>Nucleotide sequence</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>β-F</td>
<td>ATGAACCCACCTTTCCATTTCC</td>
<td>Intermediate segment amplification</td>
</tr>
<tr>
<td>β-R</td>
<td>GTACGTCACGTCTTATCG</td>
<td></td>
</tr>
<tr>
<td>β-3′-F</td>
<td>CGGTGAGGAACAGTGAC</td>
<td></td>
</tr>
<tr>
<td>RrRUP2-F</td>
<td>ATGAACCCACCTTTCCATTTCC</td>
<td></td>
</tr>
<tr>
<td>RrRUP2</td>
<td>CTAATCCGAAGCTAATGACT</td>
<td></td>
</tr>
<tr>
<td>Actin-F</td>
<td>CACTTAGACCTTCAGCAAGATG</td>
<td></td>
</tr>
<tr>
<td>Actin-R</td>
<td>CTACAACAGCAGCTAGTTTACT</td>
<td></td>
</tr>
<tr>
<td>RrRUP2-Q-F</td>
<td>ACTCTCTCCACCGTGTCC</td>
<td>qRT-PCR for RrRUP2 and Actin</td>
</tr>
<tr>
<td>RrRUP2-Q-R</td>
<td>CTCCCCCTCGTAGAACAGT</td>
<td></td>
</tr>
<tr>
<td>RrRUP2-S-F</td>
<td>ACTGTATGAACCCACCTTTTCCATTTCC</td>
<td>Expression vector construction for RrRUP2</td>
</tr>
<tr>
<td>RrRUP2-P-R</td>
<td>CACGTGCTAATCCGAAGCTAGTTTACT</td>
<td></td>
</tr>
</tbody>
</table>

2.2.3. Bioinformatics
Homologous sequence alignment analysis was performed using Blast online provided by NCBI. The open reading frame for Wd40 gene cDNA was found online, using ORF Finder predict protein secondary structure, using online software Prot-Param and CD-Search was used to analyze the physical and chemical properties of proteins and the prediction of conservative structural domains. Build the phylogenetic tree using MEGA5.0 software.

2.2.4. Expression Analysis of RrRUP2 in Different Tissues and Different Flower Developments
According to the CFX96 Real-Time System Real Time quantitative PCR and SYBR® Premix Ex Taq™ kit instructions (TaKaRa, Inc., Japan). Referencing to abm reverse transcriptase kit to synthesize cDNA and use it as template for real-time fluorescence quantitative PCR method to detect the expression of Wd40 gene in 5 developmental stages and 7 tissue sites. According to the sequence information of Wd40 gene cloned, the primers were designed by using DNAMAN software (Table 1). The reaction volume was comprised of 20 µL containing 10 µL SYBR® Premix Ex TaqTM, 0.4 µL primer (RrRUP2-Q-F and RrRUP2-Q-R) and 1 µL cDNA, with ddH2O added to a total volume of 20 µL. The reaction conditions were as follows: pre-heating at 94°C for 5 min; 39 cycles at 95°C for 10 s, at 60°C for 30 s. Signals were monitored by the Chromo 3 real-time PCR system, finally 30 s at 60°C and 30 s at 95°C for the melting curve. Each gene was set to repeat three times, and the experimental data were processed by the method of marking. Each gene was assessed with three biological replications. The relative expression levels of the genes were calculated by the 2-ΔΔCt method.
2.2.5. Construction of Expression Vector

According to the ORF of RrRUP2, the corresponding primers RrRUP2-s-f and RrRUP2-p-r (Table 1) were designed. After the sequencing validation, select right sequence to extract plasmids. The RrRUP2 was digested by SpeI and PmlI and connected to the vector pCAMBIA1304 by Solution-I. The diagram of pCAMBIA1304 was shown in Figure 1.

3. Results and Analysis

3.1. Cloning and Sequence Analysis of RrRUP2

A Wd40 protein was cloned from R. rugosa “Zi zhi” petals, named RrRUP2. The RrRUP2 gene 3’ terminal sequence of 553 bp length by using nested PCR method (Figure 2(a)) and the full length of the gene was 1173 bp (Figure 2(b)), and the open reading frame was 717 bp, encoding 390 amino acids. BLAST the nucleotide sequences and the translated amino acid sequences were compared on NCBI, and it was found that the amino acid sequences encoded by RrRUP2 were as much as 90% homologous with the RUP2 proteins of Rosa chinensis, Fragaria and homologous with the Pyrus, Malus domestica, Juglans respectively 69%, 70%, 66%. To sum up, this gene is highly cognate with the discovered RUP2 gene, which can be concluded to be RrRUP2 gene in R. rugosa.

3.2. Bioinformatics Analysis of RrRUP2 Gene

The RrRUP2 gene encodes 390 amino acids, and the predicted molecular formula is C_{3415}H_{5659}N_{1173}O_{1434}S_{313}, the relative molecular weight is 96,129.27 Da, and...
Figure 2. The results of 3’RACE amplification and full-length CDS amplification of the RrRUP2 gene. (a) 3’RACE amplification product of the RrRUP2 gene. (b) Full-length CDS amplification product of the RrRUP2 gene.

the theoretical isoelectric PI value is 5.00. Among the 390 amino acids coded, 34 basic amino acids (Arg + Lys) and 50 acidic amino acids (Asp + Glu) are included. Its instability index is 48.76, belonging to the unstable protein; The total average hydrophobic index was −0.364, belonging to hydrophilic protein. The secondary structure of RrRUP2 demonstrates that there are 10α-helix, 45 β-helix, 181 random coil, 154 extended peptide chain. The phosphorylation site prediction results reveals that there are 37 Ser phosphorylation sites, 43 Gly phosphorylation sites, 39 Val phosphorylation sites, and 29 Leu phosphorylation sites, so we can infer that it may participate in phosphorylation control.

3.3. Construction of Plasmid for Transient Gene Expression Assay

DNAman analysis shows that RrRUP2 gene is highly homologous to the Rosa chinensis (xp-024192945), genetic homology with Fragaria (xp-004309213), Pyrus (xp-009340009), Malus (xp-008376565), Juglans (xp-018830865) are respectively 85%, 69%, 70%, 66%. According to the NCBI, we found 72 RUP2 genes with similarity. Predicting the conserved domain of RrRUP2 protein amino acid sequences, the results showed that the amino acid sequences of RrRUP2 protein had typical WD and XR dipeptide structures, in which can interact with DDB1 to form an E3 ligase with CUL4 as the backbone to participate in biological processes (Figure 3).

In order to study the evolutionary relationship between RrRUP2 protein and other WD40 protein, we constructed phylogenetic trees, which showed that RrRUP2 was closely related to the RUP2 families (Figure 4).

3.4. Expression Analysis of RrRUP2 in Different Tissues and Different Flower Developments

To analyze the specificity of RrRUP2 gene in different parts and varieties, RT-pcr analysis was used to detect the transcription level of the gene in the root, stem, leaf, petals, pistils, stamens, sepals of the R. rugosa “Zi zhi”. Real-time
Figure 3. Sequence alignment between *RrRUP2* protein and other homologous proteins.

Figure 4. Phylogenetic tree of *RrRUP2* and Wd40 members from other plant species. The tree was constructed by neighbor-joining method using MEGA 5.0 software. Branch numbers represent as percentage of bootstrap values in 1000 sampling replicates and scale indicates branch lengths.

fluorescence quantitative results of gene *RrRUP2* showed (Figure 5): The highest expression level of *RrRUP2* was observed in root and sepal, while it expressed slightly in flower, pistil and leaves, and almost didn’t express in stem and stamen. The expression of this gene in five different periods of *R. rugosa* “Zi zhi”, *R. rugosa* “Bai Zi zhi” and *R. rugosa* “Fen Zi zhi” was analyzed. We found that the general trend at the bud stage, the initial stage, the half stage, the blooming stage and the end of the blooming period was that the expression of the gene decreased with the development of color. It is obvious that the expression of the *R. rugosa* “Bai Zi zhi” is much higher than that of the other two species (Figure 6).

3.5. Construction of Plasmid for Transient Gene Expression Assay

After the expression vector pCAMBIA1304 was cut with the restriction endonuclease with Spe I and PmlI (Figure 4), the *RrRUP2* gene was successfully
Figure 5. The relative expression of RrRUP2 in seven different tissues of R. rugosa “Zi zhi”. RrGAPDH was used as the internal control. The experiment was repeated three times with similar results.

Figure 6. The temporal and spatial expression patterns of RrRUP2. The relative expressions of the RrRUP2 gene in five flowering stages of R. rugosa “Zi zhi”, R. rugosa “Fen Zi zhi” and R. rugosa “Bai Zi zhi”. S1: bud stage; S2: initial stage; S3: half stage; S4: blooming stage; S5: end of the blooming period.

connected with the vector cutting through the solutionI to form a pCAMBIA1304-RrRUP2 (Figure 7). A represented the target band of RrRUP2, the light band was located near 1173 bp, which was the target gene band. B represented the target band of pCAMBIA1304. Finally, we obtain the agrobacterium vector and intend to transfer the recombinant plasmid RrRUP2-pCAMBIA1304 to Arabidopsis thaliana to verify its function.
4. Discussion

In this study, *RrRUP2* was cloned from *Rosa rugosa*. *RrRUP2* belongs to *WD40* super family. *WD40* has a variety of biochemical and cellular biological functions, mainly including signal transduction, RNA processing vesicle transport, cytoskeleton assembly and a variety of biological processes [17]. For example, it can form *MBW* complex with *MYB* and *BHLH* to regulate anthocyanin biosynthesis [18]. *RUP2* acted on the downstream of *UVR-8-cop1* and played a negative feedback regulating role in UV-B induced photomorphogenesis of plants. Through bioinformatics analysis, it was found that C-terminal conservative relatively. The 27th amino acid of C-terminal can interact with *UVR8*. In yeast, *RUP2* can interact with monomer form of *UVR8<sup>W285A</sup>*, also it can interact with dimer form of *UVR8<sup>W285F</sup>* [19]. The results showed that the amino acid sequences *RrRUP2* protein had typical WD and XR dipeptide structures, in which can interact with DDB1 to form an E3 ligase with CUL4 as the backbone to participate in biological processes [20].

The *RrRUP2* amino acid sequences cloned from *Rosa* were compared with the amino acid sequences of other species, which shows that *RrRUP2* is more than 90% similar to *RUP2* protein in *Rosa chinensis* and *Fragaria*. *RUP 1* and *RUP2* encode two highly homologous DWD proteins from two genes without introns, with protein lengths of 385 and 368 amino acids, and homology of 63 percent in 349 overlapping amino acids. From a phylogenetic perspective, these two proteins are most similar to *COP1* and *SPA* proteins in photomorphogenesis [21]. They are all important members of the UV-B signaling pathway.

The expression levels of the *RrRUP2* gene during flower development and in...
different tissues were investigated. It has been found that in the three varieties, gene expression decreased with color increasing. It is shown that RUP2 may be a negative regulator in flower and color regulation. According to the fluorescence quantification of tissues, the highest expression level of RrrRUP2 was observed in root and sepal, followed by flowers. RUP2 could regulate the expression of RUP2 gene by regulating the plant biological clock, which was called EFO2.

Light is one of the main environmental factors affecting the biosynthesis of anthocyanin [22], UV-B light is potentially destructive to both genetic material and photosynthetic systems. Plants respond to low levels of UV-B radiation and have a coordinated photoresponse that adapts to this environmental pressure factor [23]. Key participants in this UV-B reaction are COP1 (E3 ubiquitin ligase), UVR8 (propeller protein) and HY5 bZIP transcription factor) [24]. In order to prevent the over-amplification and output of UV-B signal, there is a very accurate negative feedback regulation mechanism in the plant to participate in the UV-B photomorphic signal pathway, among which RUP2 is an important negative regulator. RUP2 can promote the dimerization of UVR8, thus promoting its inactivation back to the ground state, effectively terminating the continued output of UV-B signal, and the re-formed UVR8 dimer weight has new UV-B response capacity [25]. UV-B can induce the expression of CHS, a key enzyme in the flavonoid synthesis pathway, thus promoting the accumulation of anthocyanin. However, when overexpression of RUP2 was detected, the CHS gene expression was inhibited, thus hindering the synthesis of anthocyanin.

5. Conclusion
According to the current progress, although the modified gene has been cloned from Rosa rugosa and we were going to transgenic, the regulatory mechanism of RrrRUP2 and UVR8 gene in rugosa has not been determined. It is not clear how the optical signal can be transmitted and regulated by RrrRUP2 gene in rosa under different light quality and light conditions. Recently, more and more studies have been conducted on the key genes in the anthocyanin pathway and the mechanism of flower color regulation has been improved. However, few studies have been conducted on the light which is most important environmental regulator. In this study, we cloned RrrRUP2 and analyzed its expression, which was beneficial to analyzing of how light is transmitted through signal transduction and regulating the expression of transcription factors and structural genes, and how the transcription factors involved in anthocyanin synthesis interact synergistically to the regulation; what is more, it also provided some important information to research anthocyanin in Rosa rugosa.

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


