

Cloning and Expression Analysis of *RrGT2* Gene Related to Anthocyanin Biosynthesis in *Rosa rugosa*

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Abstract

At present, the research about flower color of *Rosa rugosa* is a very innovative and practical study. Glycosylation modification fulfills an important role in increasing the stability and solubility of anthocyanin in plants. In this study, based on the transcriptional database of *R. rugosa*, a gene with full length cDNA of 1422bp, encoding 473 amino acids, designated as *RrGT2*, were isolated from flowers of *R. rugosa* 'Zizhi' and then functionally characterized. According to online software prediction, the molecular formula of the protein encoded by the *RrGT2* gene is $C_{2334}H_{3628}N_{602}O_{711}S_{18}$, the relative molecular mass is 52,075.17 Da, and the theoretical isoelectric point is $pI = 4.76$. The result of the *RrGT2* protein 3D model construction showed that it had the highest homology with the UDP-glycosyltransferase 74F2 protein model in the database (39.53%). Sequence alignments with the NCBI database showed that the *RrGT2* protein is a member of the GTB superfamily. Homology analysis revealed that the coding regions of *RrGT2* was highly specific among different species, but still had typical conserved amino acid residues called PSPG that are crucial for *RrGT2* enzyme activity. *RrGT2* transcripts were detected in five flowering stages and seven tissues of *R. rugosa* 'Zizhi', *R. rugosa* 'Fenzizhi' and *R. rugosa* 'Baizizhi', and their expression patterns corresponded with the accumulation of anthocyanins. Therefore, we speculated that glycosylation of *RrGT2* plays a crucial role in anthocyanin biosynthesis in *R. rugosa*.

Keywords

Rosa rugosa, *RrGT2* Gene, Clone, Gene Expression, Anthocyanin

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1. Introduction

Rosa rugosa is an important ornamental plant which belongs to the genus *Rosa* in the family *Rosaceae*. It is native to China and is widely distributed in the world. Because of its unique fragrance, color, cold resistance and drought resistance, it has great development potential in garden application [1]. There are many varieties of roses, but most of them are traditional colors such as pink, purple, etc. A few varieties are white, lacking yellow, bright red, orange and compound color, etc. [2]. Therefore, how to innovate rose color has become the main goal of breeders. The analysis of the pigment composition of rose and the study of the expression characteristics of the key enzymes encoding genes that catalyze the synthesis of rose pigment are the important prerequisite for rose color oriented molecular breeding [3]. Anthocyanin determines the color of higher plant organs. Its biosynthesis pathway related structural genes (*CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, *3GT* etc.) and regulatory genes (*MYB*, mostly *R2R3 MYB*, *BHLH* and *WD40* classes) have been cloned, sequenced and protein function studies in many plants, such as petunia, maize, snapdragon and so on. But less research has been done on *R. rugosa*.

Anthocyanins, derived from the anthocyanin biosynthesis pathway, are the largest group of water-soluble plant flavonoids found in organs of plants and crops [4] [5]. Anthocyanins are unstable in plants, mainly in the form of glycosides in the vacuole [6]. Anthocyanins play an important role in insect pollination, auxin transport, protection of leaves from ultraviolet radiation, inhibition of diseases and insect pests, etc. [7]. In addition, as a safe, non-toxic natural food pigment, anthocyanins also have anti-oxidation, anti-cancer and anti-arteriosclerosis functions [8].

Anthocyanin biosynthesis pathway is one of the secondary metabolic pathways in plants. At present, the research on it has been more clear. Firstly, naringin was formed by the catalysis of *CHS* and *CHI* with coumaroyl coenzyme A and malonyl Co A. Then flavonols were formed under the action of *F3H*. The next step is to form colored anthocyanins under the action of *DFR* and *ANS*. Finally, through glycosylation, methylation, acylation, hydroxylation and other modifications, a variety of anthocyanins with a stable structure were formed [9]. Flavonoid 3-O-glycosyltransferase (*3GT*) gene is a downstream gene in anthocyanin synthesis pathway. It can catalyze the glycosylation of UDP glucose to replace the 3 hydroxyl groups of anthocyanin and make anthocyanin glycosylation to produce colored and stable anthocyanins. And move the maximum absorption spectrum to the ultraviolet end, thus increasing the blue tone of anthocyanins [10]. Glycosylation can change the hydrophilicity, biochemical activity and sub-cellular localization of anthocyanins, which is beneficial to the transport and storage of anthocyanins in cells and organisms [11].

Some studies have shown that the anthocyanin content of plants lacking *3GT* also decreased significantly [12]. *3GT* gene belongs to a glycosyltransferase (GTs) family 1, whose enzyme protein has a conserved domain of about 44

amino acids at its C-terminal, known as plant secondary product glycosyltransferase (PSPG) box. At present, 3GT has been cloned and analyzed in many plants such as *Zea mays* [13], *Vitis vinifera* [14], *Gentiana triflora* [15], *Petunia hybrida* [16] and so on, which has laid a foundation for understanding the metabolic regulation of anthocyanin synthesis pathway.

At present, the studies on *R. rugosa* are mainly focused on morphological classification, geographical distribution, essential oil extraction and food quality evaluation, and there are few reports on the anthocyanin biosynthesis mechanism, so we don't know exactly how it works. In this study, based on the *R. rugosa* transcriptome data, we cloned and identified *RrGT2* gene from the petals of *R. rugosa* 'Zizhi' for the first time. We carried out detailed bioinformatics analysis, homology analysis and the temporal and spatial expression pattern analysis of the *RrGT2* gene in order to provide some useful informations for the subsequent color improvement project in *R. rugosa*.

2. Materials and Methods

2.1. Plant Materials

For *R. rugosa*, the plant materials were the same as the varieties used for transcriptome data determination. Three varieties (*R. rugosa* 'Zizhi', *R. rugosa* 'Fenzizhi' and *R. rugosa* 'Baizizhi') cultivated in Rose germplasm nursery of Shandong Agricultural University was used as the test material. All three varieties were crossed with *Rosa davurica* as female parent and *Rosa rugosa* Thunb. as male parent. We collected the petals at the budding stage, initial opening stage, half opening stage, full opening stage and wilting stage in the forenoon On sunny days from 20 April to 10 May 2017. Seven tissues (roots, stems, leaves, petals at the budding stage, sepals, stamens and pistil) of *R. rugosa* 'Zizhi' were collected at the same time. After quick freezing of liquid nitrogen, all samples collected with three replicates were put into -80°C refrigerator for storage.

2.2. Extraction of Total RNA and Synthesis of the First-Strand cDNA

The extraction of total RNA is based on the specification of EASY spin plant RNA rapid extraction kit (Aidlab Biotech, Beijing, China). The integrity of RNA was detected by gel electrophoresis with 1.0% nondenatured agarose, the purity and concentration of RNA were detected by Nanodrop2000C ultramicro spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA), and the qualified RNA was preserved at -80°C . The first-strand cDNA was synthesized by referring to the steps of 5 × All-In-One RT MasterMix reverse transcription kit (ABM Company, Vancouver, Canada) and synthesized according to the requirements of RT-PCR and qRT-PCR.

2.3. Full-Length CDS Cloning of the *RrGT2* Gene

3'RACE specific primers were designed based on the sequence information pro-

vided by the *Rosa* transcriptome data. The cDNA 3'terminal sequence of the target gene was amplified by 3'RACE technology. The known target gene sequence in transcriptome data and the 3'terminal sequence obtained by RACE technique were spliced with DNAMAN software to obtain the full-length cDNA sequence of the *RrGT2* gene. According to the sequence obtained by splicing, the upstream primer *RrGT2*-F containing the starting codon of the *RrGT2* gene and the downstream primer *RrGT2*-R containing the terminating codon (**Table 1**) were designed and amplified. The estimated length of amplification was 1422bp.

2.4. Bioinformatics Analysis of the *RrGT2* Gene

Bioinformatics analysis softwares and tools were used to predict the physico-chemical properties and structural functions of the protein encoded by the *RrGT2* gene, which provided a reference for the future research and application of the gene. The basic physical and chemical properties of the protein encoded by the *RrGT2* gene were analyzed by using Prot-Param tools in ExPasy (<http://web.expasy.org/protparam/>). The CD-Search function (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) on NCBI was used to predict the conserved domain in the protein encoded by the *RrGT2* gene. The 3D structure of the *RrGT2* protein was predicted by online tool SWISS-MODEL. DNAMAN software and Blast tool in NCBI were used to analyze the amino acid sequence of the *RrGT2* gene. The phylogenetic tree is constructed by neighbor-joining method using MEGA5.0 software.

2.5. qRT-PCR Detection

The gene expression was analyzed by qRT-PCR on a Bio-Rad CFX96™ Real-Time PCR instrument (Bio-Rad, Inc., USA). The qRT-PCR mixture (20 μ L total volume) contained 10 μ L of SYBR® Premix Ex Taq™ (TaKaRa, Inc., Japan), 8.2 μ L of ddH₂O, 0.4 μ L of each primer and 1 μ L 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, 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 replications. The relative expression levels of the genes were calculated by the $2^{-\Delta\Delta C_t}$ method [17].

3. Results

3.1. Cloning of *RrGT2* and Sequence Analysis

We obtained the *RrGT2* gene 3' terminal sequence of 236 bp length by nested PCR (**Figure 1(a)**). The full length CDS sequence (**Figure 1(b)**) of the *RrGT2* gene was cloned by full-length primers (**Table 1**) and confirmed by sequencing. The *RrGT2* gene has a complete ORF from the starting codon ATG to the termination codon TAA, encodes a 473-amino acids protein and has a polyA tail. Sequence alignments with the NCBI database showed the *RrGT2* protein is a member of the GTB superfamily and has a typical plant secondary product glycosyltransferases conserved domain consisting of 44 amino acid residues at the

Table 1. Primers used in the present study.

Primer Name	Sequence(5'-3')	Description	Annealing temperatures/°C
3' <i>RrGT2</i> -F-outer	GCAGAGAGGAACTAGAAGAGCTTGGG		61.2
3' <i>RrGT2</i> -F-inner	GCCAAGTTGATAGAGGACATGTGG		60.4
B26	GACTCGAGTCGACATCGATTTTTTTTTTTTT	3'RACE for <i>RrGT2</i>	56
<i>RrGT2</i> -F	TTTT ATGACGCAACACCGCTTC		56
<i>RrGT2</i> -R	CTAGGCTGGCAAACAGCCA	Full-length cDNA for <i>RrGT2</i>	60
<i>RrGAPDH</i> -F	TTCTGCCTGCTCTCAATG		52.1
<i>RrGAPDH</i> -R	TGCCTTCTTCTCAAGTCTG	qRT-PCR for <i>RrGAPDH</i>	51.5
q <i>RrGT2</i> -F	GTTGTTGGATTCCGGACGTC		57.5
q <i>RrGT2</i> -R	CAAGCTCTTCTAGTTCCTCTCTGC	qRT-PCR for <i>RrGT2</i>	60.4

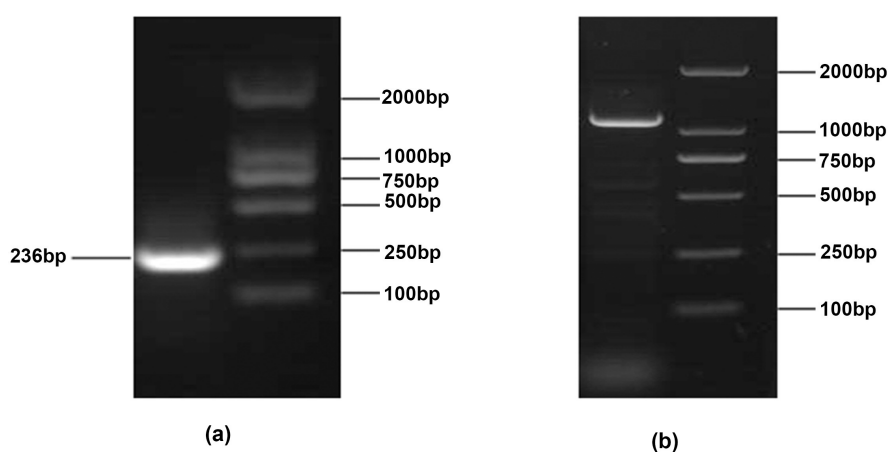


Figure 1. The results of 3'RACE amplification and full-length CDS amplification of the *RrGT2* gene. (a) 3'RACE amplification product of the *RrGT2* gene. (b) Full-length CDS amplification product of the *RrGT2* gene.

C-terminal. According to online software prediction, the molecular formula of the *RrGT2* protein is $C_{2334}H_{3628}N_{602}O_{711}S_{18}$, the relative molecular mass is 52075.17 Da, and the theoretical isoelectric point is $pI = 4.76$.

3.2. Protein 3D Model Construction

During the construction of the *RrGT2* protein 3D model, templates search with BLAST and HHblits has been performed against the SWISS-MODEL template library. It was found that it had the highest homology with the UDP-glycosyltransferase 74F2 protein model in the database (39.53%), so the *RrGT2* protein 3D model (**Figure 2**) was constructed on the basis of its model.

3.3. Homology Analysis

Sequence alignment of multiple species amino acids (**Figure 3**) showed that the *RrGT2* protein has strong species specificity in the N-terminal region and PSPG conserved domain in the C-terminal region. Using MEGA5.0 software,

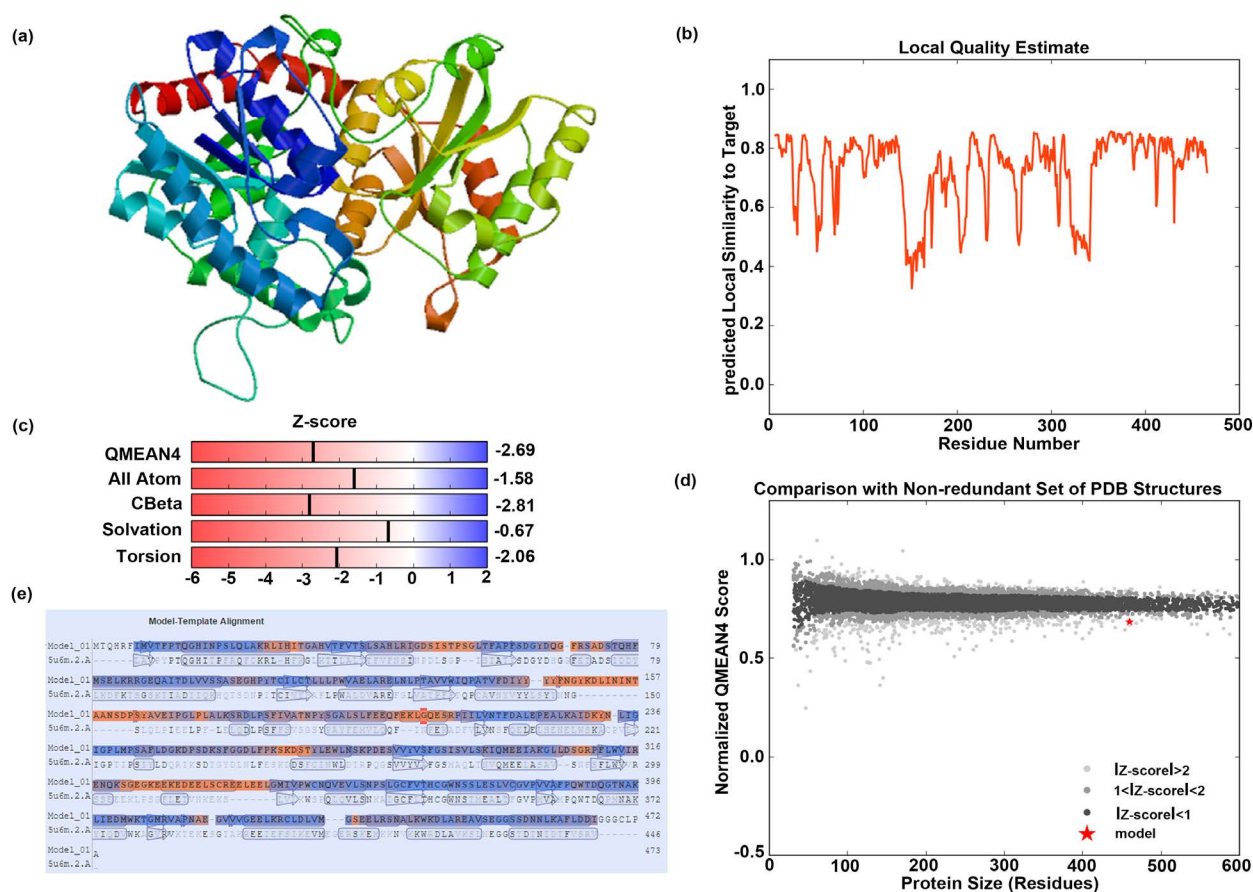


Figure 2. The protein modelling results and quality estimation of *RrGT2*. (a) Visualizable 3D model of *RrGT2*. (b) Local quality estimation of the protein. (c) As a statistical value, Z-score can be used to represent the matching degree between template proteins and proteins to be tested. QMEAN: A comprehensive scoring function for model quality assessment. All Atom: Normalized all-atom potential energy of the residue calculated from the short-range statistical potentials. CBeta: Normalized cbeta potential energy of the residue calculated from the short-range statistical potentials. Solvation: Normalized solvation potential energy of the residue calculated from the short-range statistical potentials. Torsion: Torsion energy of the residue exposed relative solvent accessibility, calculated by dividing the maximally accessible surface area (ASA) of a residue by the observed value. (d) Comparison with Non-redundant Set of PDB (Protein Data Bank) structures. (e) Alignment between the model and the template.

phylogenetic tree (Figure 4) was constructed from 21 plant amino acids sequences including *R. rugosa RrGT2*. The results showed that the *RrGT2* gene had the closest genetic relationship with *FaUGT* and *FvGT*, and its homology reached 89%.

3.4. Temporal and Spatial Expression Patterns of the *RrGT2* Gene

The expression levels of the *RrGT2* gene, which significantly differed, were assessed during five flowering stages. For *R. rugosa* 'Zizhi' (Figure 5(a)), the highest expression level was observed during the full opening stage, and the lowest was observed during the budding stage. For *R. rugosa* 'Fenzizhi' (Figure 5(b)), the highest expression level was observed during the wilting stage, and the lowest was observed during the budding stage. And for *R. rugosa* 'Baizizhi' (Figure 5(c)), the expression level was highest during the initial opening stage but lowest

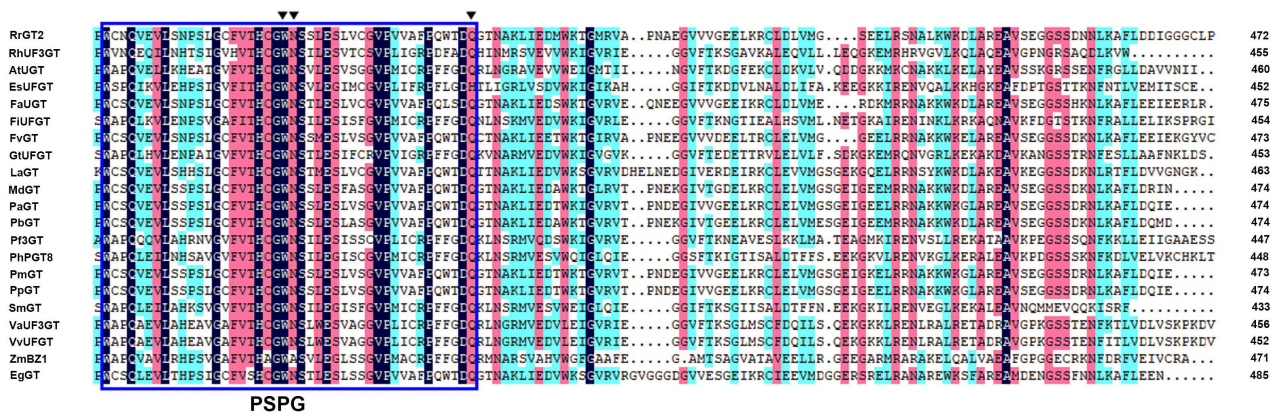


Figure 3. Amino acid sequences homologous analysis of the *RrGT2* gene and other species related genes. Alignments were performed using DNAMAN. The relative genes of 21 species including the *RrGT2* gene were selected for comparative analysis. The blue box shows the PSPG domains. Black triangles (from left to right) indicate 22, 23 and 44 amino acids in the PSPG box.

also during the budding stage. The expression patterns of the *RrGT2* gene in *R. rugosa* ‘Zizhi’, *R. rugosa* ‘Fenzizhi’ and *R. rugosa* ‘Baizizhi’ showed different trends.

Then the expression levels of the *RrGT2* gene in three varieties was compared in five flowering stages (Figure 5(d)). In the budding stage, full opening stage and wilting stage, *R. rugosa* ‘Fenzizhi’ > *R. rugosa* ‘Baizizhi’ > *R. rugosa* ‘Zizhi’, while in the initial opening stage and half opening stage, the trends of gene expression in the three varieties were consistent: *R. rugosa* ‘Baizizhi’ > *R. rugosa* ‘Fenzizhi’ > *R. rugosa* ‘Zizhi’.

The expression levels of the *RrGT2* gene, which also significantly differed, were assessed in seven different tissue types of *R. rugosa* ‘Zizhi’ (Figure 5(e)). The order of relative expression levels of the *RrGT2* gene in seven tissues was: sepal > leaf > stem > root > flower at the budding stage > pistil > stamen.

4. Discussion

Although many genes have been reported to regulate the formation of flower color, there are few reports of downstream structural genes such as GTs. The final formation of anthocyanins depends on the glycosylation of GTs, so it is very important to elucidate the function and influence of the *RrGT2* gene in *R. rugosa* color formation. In this study, we successfully cloned *RrGT2* gene with full length cDNA of 1422bp, encoding 473 amino acids from the petals of *R. rugosa* ‘Zizhi’. It was predicted that the molecular formula of the protein encoded by the *RrGT2* gene is $C_{2334}H_{3628}N_{602}O_{711}S_{18}$, the relative molecular mass is 52,075.17 Da, the theoretical isoelectric point $pI = 4.76$, which belongs to GTB superfamily. During the construction of the *RrGT2* protein 3D model, it was found that the *RrGT2* protein had the highest homology with the existing UDP-glycosyltransferase 74F2 protein model protein model (39.53%), which confirmed the function of the *RrGT2* gene to some extent.

The evolutionary analysis of flavonoids GTs by Sawada *et al.* [18] showed that GTs, which catalyze the glycosylation of flavonoids in different positions

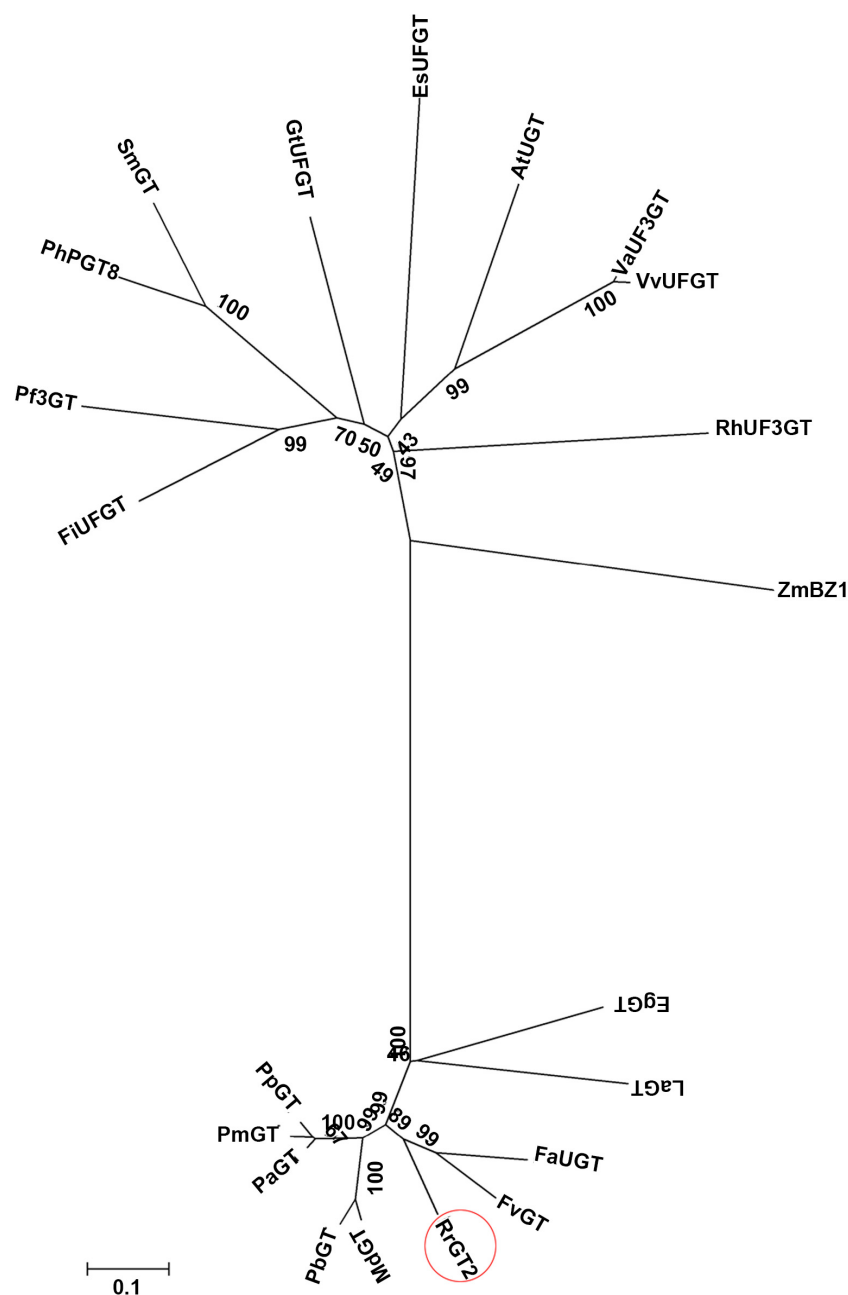


Figure 4. Phylogenetic tree of *RrGT2* and GT members from other plant species. The tree was constructed by neighbor-joining method using MEGA 5.0 software. Branch numbers represent as percentage of bootstrap values in 1000 sampling replicates and scale indicates branch lengths.

(3-O, 5-O, 7-O), were formed into different evolutionary branches (F3Gly T, F5Gly T, F7Gly T) without restriction of species. It suggested that the region-specific of flavonoids GTs to glycosyl receptors (catalytic site specificity) was formed before species differentiation. In the course of evolution, the ability to utilize UDP-sugar (UDP-glucose, UDP-rhamnose, UDP-galactose) was obtained. Phylogenetic tree analysis showed that *RrGT2* was linked to *FaUGT* and

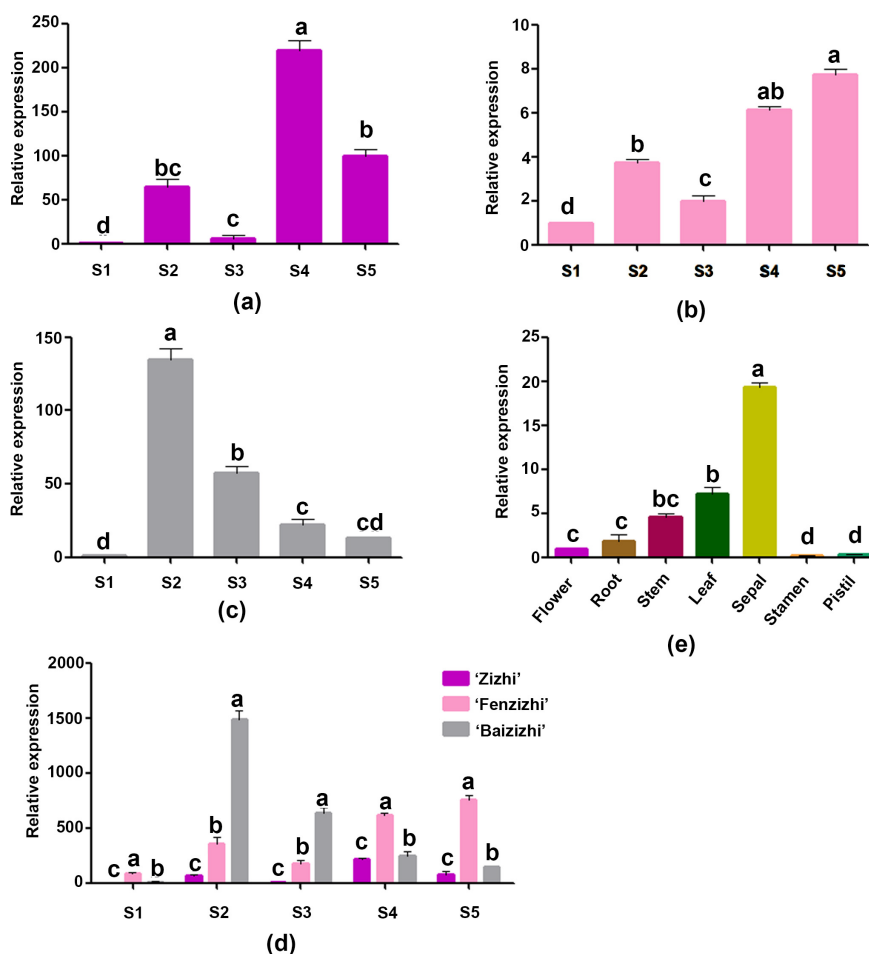


Figure 5. The temporal and spatial expression patterns of *RrGT2*. The relative expressions of the *RrGT2* gene in five flowering stages of *R. rugosa* 'Zizhi' (a), *R. rugosa* 'Fenzizhi' (b) and *R. rugosa* 'Baizizhi' (c). S1, budding stage; S2, initial opening stage; S3, half opening stage; S4, full opening stage; S5, wilting stage. (d) Comparison of relative expressions of *RrGT2* in five flowering stages of three varieties above. (e) The relative expression of *RrGT2* in seven different tissues of *R. rugosa* 'Zizhi'. *RrGAPDH* was used as the internal control. Error bars represent the SDs of triplicate reactions. The experiment was repeated three times with similar results. Different lower case letters represent significant difference which is calculated using LSD analysis at the level of $P < 0.05$.

FvGT of 3-O-glycosylation of flavonoids, suggesting that *RrGT2* may be involved in the glycosylation process of 3-O positions of flavonoids.

The alignment of amino acid sequences between *RrGT2* and glycosyltransferases from the other 21 species indicated that *RrGT2* possessed a common PSPG motif of the glycosyltransferases superfamily (Figure 3). Previous studies have shown that the conserved region of PSPG is related to substrate recognition and catalytic activity of enzyme proteins [19] [20] [21] [22]. If the 44 amino acids of the PSPG domain are numbered, the amino acids at position 22, 23 and 44 play an important role in the selection of enzyme proteoglycan donors. The twenty-second position of tryptophan (Trp, W) can correctly locate UDP-glucose, while arginine (Arg, R) can make UDP-glucuronic acid locate correctly; the

twenty-third position of serine (Ser, S) is highly conserved in UDP-glucuronosyltransferase [23] [24] [25]; the forty-fourth glutamine (Gln, Q) and histidine (His, H) have strong conservatism in glucosyltransferase and galactotransferase respectively [22]. In the PSPG domain of the *RrGT2* gene, the amino acids at position 22, 23 and 44 are tryptophan (Trp, W), asparagine (Asn, N) and glutamine (Gln, Q), respectively. Therefore, we speculated that the *RrGT2* gene is using UDP-glucose as the main glycosyl donor, but has no glucuronosyltransferase activity.

The expression levels of the *RrGT2* gene during flower development and in different tissues were investigated. It was found that the expression of the *RrGT2* gene showed a different trend during different flowering periods in three *R. rugosa* varieties, indicating that the expression of the *RrGT2* gene was developmentally regulated in the process of anthocyanin biosynthesis. Studies have shown that the accumulation of anthocyanin in red skinned sand pear, strawberries and litchi is positively correlated with the activity of *UF3GT*. Boss *et al.* [26] also detected the expression of *UF3GT* in the peels of red grape which accumulated anthocyanin, but not in other tissues of red grape and white grape without anthocyanin accumulation. Gong *et al.*'s [27] studies showed that the partial structural genes of *Perilla frutescens* anthocyanin metabolic pathway were only expressed in the leaves of red varieties, but not in green varieties or the expression in the green leaves was very low. About the tissue-specific expression in *R. rugosa* 'Zizhi', it is worth mentioning that the stems of *R. rugosa* 'Zizhi' are purple, which is consistent with the relatively high level of expression of the *RrGT2* gene. But for the relatively low level of expression in flowers, this did not mean that the *RrGT2* gene had no effect on flower color formation. We believe that this was the result of comparing flowers at the budding stage as tissue types. Because in the budding stage, the expression of the *RrGT2* gene was lowest, but in other stages was very high. In addition, *RrGT2* was also highly expressed in the sepals and leaves, so we infer that *RrGT2* is also involved in the glycosylation of secondary metabolites in sepals and leaves and plays an important role.

In conclusion, the cloning and expression analysis of the *RrGT2* gene was beneficial to analyzing the molecular synthesis and regulation mechanism of anthocyanins, and also provided some important informations for the improvement of *R. rugosa* flower color in the future.

Acknowledgements

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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