Scientific Research Publishing

American Journal of Plant Sciences, 2019, 10, 265-275
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Cloning and Expression Analysis of *TTG*1 Gene Related to *Rosa rugosa* Trichomes Formation

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How to cite this paper: Wang, Y., Zhao, M.Y., Xu, Z.D., Zhao, L.Y. and Han, X. (2019) Cloning and Expression Analysis of *TTG*1 Gene Related to *Rosa rugosa* Trichomes Formation. *American Journal of Plant Sciences*, **10**, 265-275.
https://doi.org/10.4236/ajps.2019.102020

Received: January 7, 2019
Accepted: January 31, 2019
Published: February 3, 2019

Abstract

The *TTG*1 transcription factor plays an important role in the formation of plant trichomes. Based on the *R. rugosa* transcriptome data, this study cloned a *R. rugosa TTG*1 gene, named *RrTTG*1, and carried out bioinformatics analysis and fluorescence quantitative analysis to explore the relationship between *TTG*1 gene and *R. rugosa* trichomes formation, in order to lay a good foundation to cultivate a thornless plant in the family *Rosaceae*. In this experiment, six hybrid cultivars of *R. rugosa* "Zizhi", *R. rugosa* "Xizi", *R. rugosa* "Tang fen", *R. rugosa* "Hun chun", *R. rugosa* "Zi long wo chi" and *R. rugosa* "Tian e huang" were used as experimental materials, and the cDNA full length of this gene was obtained by RT-PCR and RACE, and the full length of the cDNA was 1348 bp. After bioinformatics analysis, it is predicted that its molecular formula is $C_{1723}H_{2661}N_{465}O_{529}S_{12}$, the molecular weight is 38.71 KB, and the isoelectric point is 5.00. Its instability index is 54.30, which belongs to unstable protein; and its hydrophilic amino acid distribution is relatively uniform, and the amount is larger than hydrophobic amino acid, which belongs to hydrophilic protein. Phylogenetic tree was constructed for the *TTG*1 gene. Evolutionary analysis indicated that *RrTTG*1 is closely related to the *TTG*1 protein of *Rosaceae* family, and has a close relationship with other families. The expression analysis showed that the expression of *RrTTG*1 protein was negatively correlated with the trichome content of *R. rugosa* stems and leaves. The expression levels of the three spiny varieties of *R. rugosa* "Hun chun", *R. rugosa* "Xizi" and *R. rugosa* "Zi long wo chi" were lower, and the expressions of the three less thorn varieties of *R. rugosa* "Zizhi", *R. rugosa* "Tian e huang" and *R. rugosa* "Tang fen" were higher. According to the above results, it was speculated that *RrTTG*1 is involved in the synthesis of *R. rugosa* trichomes and belongs to the negative regulation mechanism.

Keywords

R. rugosa, Trichome, *RrTTG*1, Gene Expression

*These authors contributed equally.

1. Introduction

R. rugosa is a perennial evergreen or deciduous shrub of the genus *Rosa* in the family *Rosaceae*. It is rich in fragrant citronellol, eugenol and other aroma substances and a large number of vitamins, pigments, ecdysteroids and other substances. It can be used as a raw material for food, cosmetics, spices, pigments and medicines. And it has high economic value. At present, *R. rugosa* is getting more and more attention [1] [2] [3]. However, because the stem of the *R. rugosa* has many thorns, it has many inconveniences in the process of cultivation management and flower picking. If it can breed a few thorns or even thornless *R. rugosa*, it will greatly improve the work efficiency in the process of cultivation management and flower picking. The thorn of the *R. rugosa* belongs to the prickle, which is a special form of the epidermis. Plant trichomes are developed from epidermal cells and are a protective tissue covering the surface of plants. It is the outermost layer of plant contact with the environment. There are many different types of plant trichomes, and different species are different, even though the same species may exist in several different types [4]. In addition to prickles, the leaf hair and root hair of *R. rugosa* are also a type of trichomes.

In recent years, the research on the molecular genetic control mechanism of plant trichomes development has made great progress, but it has been concentrated on the model plant *Arabidopsis* for a long time, and the development model has been studied relatively thoroughly. Although some studies have been carried out on the morphological structure, histochemistry, developmental origin and genetic analysis of trichomes of other plants of *Rosaceae* [5] [6] [7], the understanding of molecular genetics and control of trichomes development is not deep enough [8]. Therefore, this experiment is based on the molecular regulation mechanism of *Arabidopsis* trichomes development. *R. rugosa* "Zizhi", *R. rugosa* "Xizi", *R. rugosa* "Tang fen", *R. rugosa* "Hun chun", *R. rugosa* "Zi long wo chi" and *R. rugosa* "Tian e huang" were used as experimental materials to isolate the *TTG*1 gene associated with the formation of plant trichomes, aiming to lay the foundation for systematic study of the molecular mechanism of *R. rugosa* trichomes development. *TTG*1 is one of the important genes controlling trichomes development in *Arabidopsis* [9], and its mutant *ttg*1 has a functional loss, which is manifested as almost complete loss of trichomes development; and its over expression in *Arabidopsis* wild type does not produce excessive trichomes [10]. In addition, the results of *TTG*1 gene in many other plants also indicate the importance for trichomes formation, including *Cucumis sativus* [11], *Brassica napus* [12], *Saussurea hypsipeta* [13], *Saussurea medusa* [14], *Nicotiana tabacum* [15], *Rosa roxbunghii* [16] and so on.

Based on the transcriptome of *R. rugosa*, we cloned a *TTG*1 gene associated with *R. rugosa* trichomes synthesis, named *RrTTG*1, and then analyzed its bioinformatics and constructed a phylogenetic tree to predict its function. The expression levels of *RrTTG*1 in different varieties and different tissue sites were identified by using qPCR technology. By figuring out how *RrTTG*1 works in the synthesis of *R. rugosa* trichomes, we could lay a solid foundation for the devel-

opment of new varieties of *Rosaceae* family that are less or even thornless.

2. Materials and Methods

The experiment was conducted at the flower germplasm resource nursery of Shandong Agricultural University and the Flower Institute of Forestry College.

2.1. Plant Material

We chose healthy individuals with stable floral inheritance of *R. rugosa* "Zizhi", *R. rugosa* "Xizi", *R. rugosa* "Tang fen", *R. rugosa* "Hun chun", *R. rugosa* "Zi long wo chi" and *R. rugosa* "Tian e huang" as experiment material, which grown in the field of rose germplasm resource nursery. The above six varieties of roots, stems, leaves and flowers were collected for gene cloning and differential expression tests between the varieties. All samples were collected and frozen in liquid nitrogen immediately, then stored at −80˚C until used.

2.2. Methods

2.2.1. RNA Extraction, cDNA Synthesis and Gene Cloning

The extraction of RNA from various tissue parts of *R. rugosa* was operated according to the instructions of EASYspin plant RNA rapid extraction kit, the integrity of RNA was detected by 1% agarose gel electrophoresis and its concentration and purity were tested by ultraviolet spectrophotometer. The cDNA was synthesized by RNA reverse transcription kit according to the description of abm reverse transcription kit.

Full-length amplification: The specific primers for complete ORF amplification "Table 1" were designed by DNAMAN software. The synthesized cDNA was used as template, and the reaction system was as follows: 2 × Gflex PCR Buffer 12.5 μL, the target gene upstream and downstream primers each 1 μL, template cDNA 1 μL, TKS Gflex DNA Polymerase 0.5 μL, sterilization ddH_2O supplement to 25 μL; The PCR reaction conditions are as follows: 94˚C for 1 min; 98˚C for 10 s, 55˚C for 15 s, and 65˚C for 30 s for a total of 30 cycles. The PCR products was detected by 1% agarose gel electrophoresis, and the target product was reclaimed according to the description of Hipure Gel Pure DNA Mini Kit (Magen), then ligated with the carrier pMD18-T to transform Escherichia coli DH5α. Positive clones were selected and sequenced at Ruibiotech.

2.2.2. Bioinformatics Analysis

The ORF finder (NCBI) was used to search for an open reading frame, and the Conserved Domains database (NCBI) was used to analyze the conserved domains. ExPaSy-SOPMA was used to predict protein secondary structure. We used BLASTX (NCBI) to study the homology of the nucleotide sequence and conducted multiple sequence alignment by DNAMAN, and then constructed phylogenetic tree with other anthocyanin-promoting proteins by MEGA5.0 software. The phylogenetic tree was constructed according to the neighbor-joining method, and tested by bootstrap, which was repeated 1000 times.

Table 1. Primers used to clone and analyze the expression of *RrTTG*1 and the vector construction in *Rosa rugosa*.

Primer name	(5'→3') Nucleotide sequence*	Purpose
Rr-*TTG*1-F	ATGGAGAACTCGACCCAAG	Full-length cDNA for *Rr-TTG*1
Rr-*TTG*1-R	TCAAACCTTCAACAGCTGC	
Actin-F	CACTTAGCACCTTCCAGCAGATGT	qRT-PCR for *Rr-TTG*1 and Actin
Actin-R	CTACAACAGCAGACCTGAGTTCACT	
Rr-*TTG*1-Q-F	ACTAGTATGGAGAACTCGACCCAAG	
Rr-*TTG*1-Q-R	CACGTGTCAAACCTTCAACAGCTGC	
Rr-*TTG*1-P-F	ACATCCTCGCCTCTTCCG	Expression vector construction for *Rr-TTG*1
Rr-*TTG*1-P-R	GGGCTCGATCTCGTTCCA	

2.2.3. Expression Analysis of Rr*TTG*1 in Different Varieties and Different Tissues

The cDNA synthesized from RNA in different tissues and different variety was used as template, and it was carried out following the instruction of SYBR®Premix ExTaqTM kit by CFX96TM Real-Time System RT-qPCR instrument. The suitable primers of Rr*TTG*1 for RT-PCR were selected by standard curve method. The specific primers and internal reference primers (Actin) are shown in "Table 1". The reaction system is: Mix10 ul, cDNA 1 μL, 0.4 μL each of upstream and downstream primer, adding sterilizing ddH_2O up to 20 μL. The reaction procedure was: predenaturation at 95˚C for 30 s; 95˚C for 30 s, 60˚C for 30 s for a total of 39 cycles. The relative expression level was calculated by the method of 2−ΔΔCT. And the data analysis was graphed through Sigmaplot 10.0.

2.2.4. Construction of Expression Vector

Choosing *Spe*I and *Pml*I as restriction enzymes, designing primers with restriction enzyme sites and then cloning the full length. Then the *RrTTG*1 and expression vector pCAMBIA1304 were digested by restriction endonucleases SpeI and PmlI, and the target fragments were recycled and connected with solution I. Positive clones were selected and sequenced at Ruibiotech. The diagram of pCAMBIA1304 was shown in "Figure 1".

3. Results and Analysis

3.1. Cloning and Sequence Analysis of *RrTTG*1

The *TTG*1 gene was cloned from the petals of *R. rugosa* "Zizhi", named *RrTTG*1. The full length of *RrTTG*1 gene was 1348 bp, and the open reading frame was 1038 bp "Figure 2", encoding 346 amino acids.

Multiple sequence alignment analysis revealed "Figure 3" that the amino acid sequence encoded by *RrTTG*1 has four WD40 repeat units, which, like the *AtTTG*1 gene of the model plant *Arabidopsis*, indicates that *RrTTG*1 is a homologous gene belonging to WD 40 superfamily. And the domain is a short peptide

containing about 40 amino acids, usually starting with Gly-His and ending with Try-Asp. In order to study the evolutionary relationship between *RrTTG*1 and *TTG*1 in other species, the phylogenetic tree was constructed and analyzed by BLAST alignment of 15 species with higher homology. The phylogenetic tree was constructed using MEGA 5.0 software, and the phylogenetic tree was tested using bootstrap for a total of 1000 repetitions "Figure 4". The results showed that the *TTG*1 gene was closely related to the *TTG*1 protein of the *Rosa chinensis*, and it was clustered with seven *TTG*1 proteins of other *Rosaceae* family plants such as *Fragaria vesca*, *Rubus chingii*, *Amygdalus persica*, *Malus domestica* and *Pyrus bretschneideri*. Five kinds of *TTG*1 proteins such as *Malva sinensis*, *Hevea brasiliensis*, *Morus alba*, *Ziziphus jujuba* are clustered together, which is far away from *R. rugosa*; the *TTG*1 protein of *Nicotiana tabacum* and *Solanum tuberosum* is clustered, and the relationship with the *RrTTG*1 protein is the farthest.

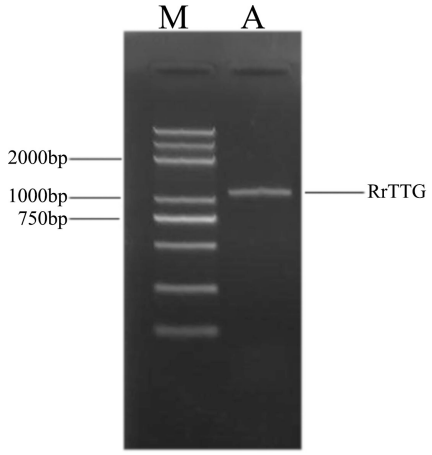

Figure 1. Diagram of pCAMBIA1304.

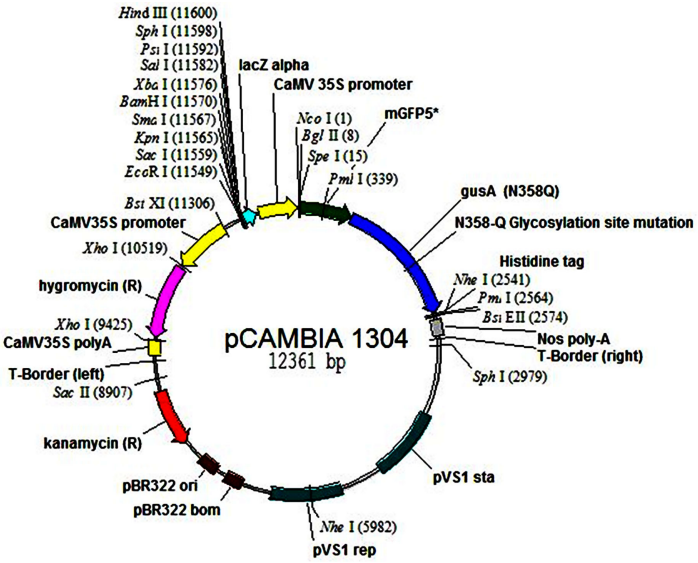

Figure 2. *RrTTG*1 gene cloning M: Marker A: *RrTTG*1.

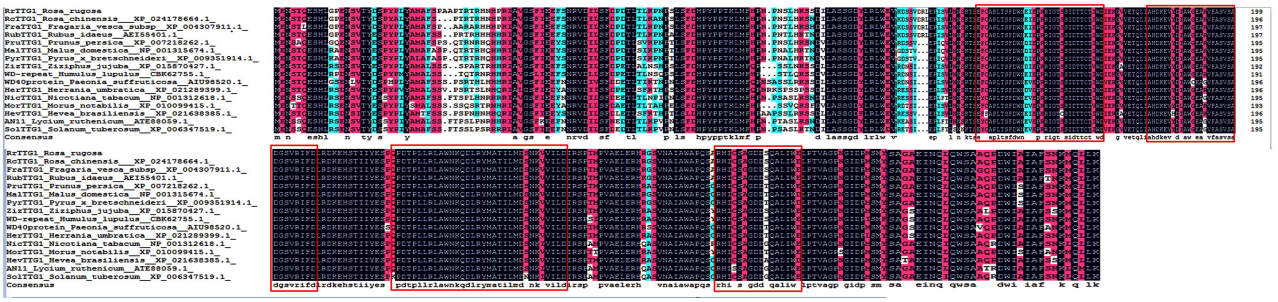

Figure 3. Multiple alignment of the *RrTTG*1 protein and *TTG*1 protein from other plants. Dark Shading indicates positions where all sequences are identical conserved. While colour shading represents positions where there is a lower level of conservation. The positions of the WD repeats are indicated with red boxes.

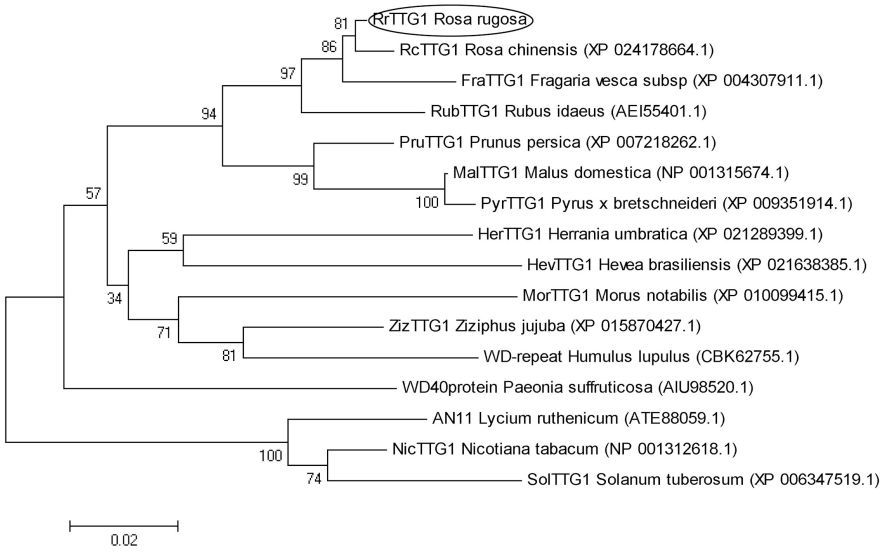

Figure 4. Phylogenetic tree analysis of *RrTTG*1 protein and *TTG*1 protein from other plants.

3.2. Bioinformatics Analysis of *RrTTG*1 Gene

There are 346 amino acids encoded by *RrTTG*1. The protein's formula is $C_{1723}H_{2661}N_{465}O_{529}S_{12}$ with a molecular weight of 38.71 KB and a theoretical pI: 500. Among the composition of the entire amino acid, acidic amino acids accounted for 13.01%, basic amino acids accounted for 11.27%, hydrophobic amino acids accounted for 44.80%, charged amino acids accounted for 21.38%, and polar amino acids accounted for 30.92%. Its instability index is 54.30, which is unstable protein. The hydrophilic amino acid distribution of *RrTTG*1 is relatively uniform and the amount is larger than that of hydrophobic amino acids. It is concluded that *RrTTG*1 protein is a hydrophilic protein. The secondary structure of *RrTTG*1 demonstrates that there are 38 α-helix, 182 random coil, 114 extended peptide chain, and 12 β-turn. The phosphorylation site prediction results reveals that there are 37 Ser phosphorylation sites, 28 Ala phosphorylation sites, and 28 Leu phosphorylation sites, so we can infer that it may participate in phosphorylation control.

3.3. Expression Analysis of *RrTTG*1 in Different Varieties and Different Tissues

To analyze the cultivar specificity and tissue specificity of *RrTTG*1 gene expression, the *RrTTG*1 gene was detected by quantitative RT-PCR in roots, stems and leaves of *R. rugosa* "Zizhi", *R. rugosa* "Xizi", *R. rugosa* "Tang fen", *R. rugosa* "Hun chun", *R. rugosa* "Zi long wo chi" and *R. rugosa* "Tian e huang". The real-time fluorescence quantitative results of *RrTTG*1 indicated that the expression level of the leaves was the highest in the different tissues of *R. rugosa* "Zizhi" "**Figure 5(a)**" and *R. rugosa* "Tian e huang" "**Figure 5(b)**", and the expression in the stem was the second, and the expression in the root was the lowest; The expression of *RrTTG*1 was highest in the leaves of *R. rugosa* "Xizi" "**Figure 5(c)**" and *R. rugosa* "Tang fen" "**Figure 5(f)**", the second in the root, and the lowest in the stem; The expression of this gene was highest in the roots of different parts of *R. rugosa* "Zi long wo chi" "**Figure 5(d)**", the expression in the stem was the second, and the expression in the leaves was the lowest. The expression of this gene in the different tissues of *R. rugosa* "Hun chun" "**Figure 5(e)**" was similar, the expression level in the stem was the highest, and the expression in the root and leaf was not much different. Comparing the expression levels of *RrTTG*1 gene in the roots "**Figure 6**" of six cultivars, it was found that the expression level of *R. rugosa* "Zi long wo chi" was the highest, followed by *R. rugosa* "Tian e huang", and the expression of *RrTTG*1 in the other four varieties was very small; The expression levels of the gene in the stems "**Figure 7**" of six varieties showed that the expression level of *R. rugosa* "Tian e huang" was the highest, the expression level of *R. rugosa* "Zizhi" and *R. rugosa* "Tang fen" was the second, and the expression level of the other three varieties was lower. Comparing the expression levels of *RrTTG*1 gene in the leaves "**Figure 8**"of six varieties, the expression level of *R. rugosa* "Tang fen" was the highest, and the expression levels of *R. rugosa* "Tian e huang", *R. rugosa* "Zizhi", *R. rugosa* "Hun chun" and *R. rugosa* "Xizi" were similar. *R. rugosa* "Zi long wo chi" has the lowest expression level.

3.4. Construction of Plasmid for Transient Gene Expression Assay

The *RrTTG*1 and expression vector pCAMBIA1304 were digested by restriction endonucleases *Spe*I and *Pml*I, and the target fragments "**Figure 9**" were recycled and connected with solution I. Enzyme digestion analysis and DNA sequencing showed that the co-expressed protein expressing vectors were constructed successfully. Next, we are going to transfer the recombinant plasmid *RrTTG*1-pCAMBIA1304 to Arabidopsis thaliana to verify its function.

4. Discussion

In this study, the *RrTTG*1 gene was cloned from *R. rugosa*, and its structural characteristics were analyzed. It was found that the *RrTTG*1 protein coding sequence has four WD40 repeating units, which is a short peptide containing about 40 amino acids, often starting with glycine-histidine, ending with trypto-

phan-aspartate. WD40 protein is a large family with extensive cell biology and biochemical functions, such as involved in photoreceptor and conduction, cell division and cytoplasmic movement, flower development, flowering, cell movement, and programmed cell death [17] [18] [19].

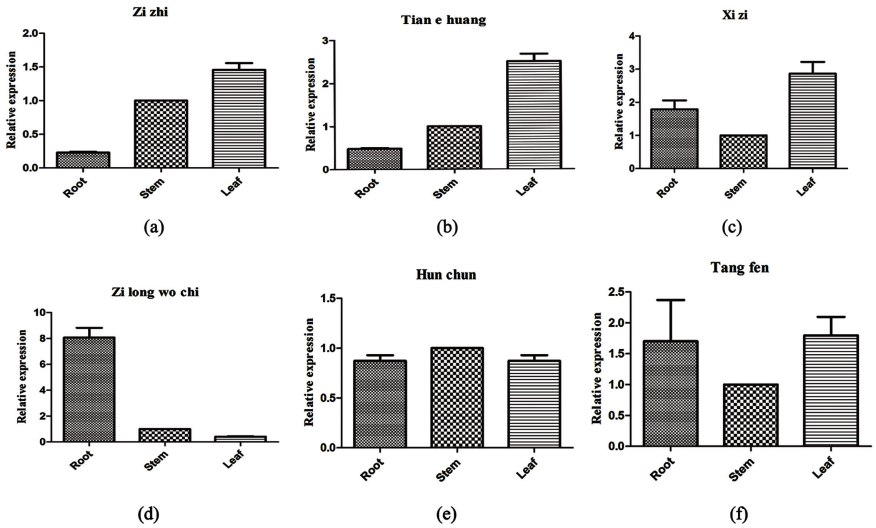

Figure 5. Relative expression of *RrTTG*1 in different varieties.

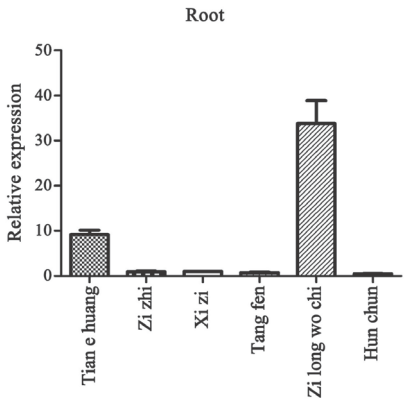

Figure 6. Relative expression of *RrTTG*1 in roots.

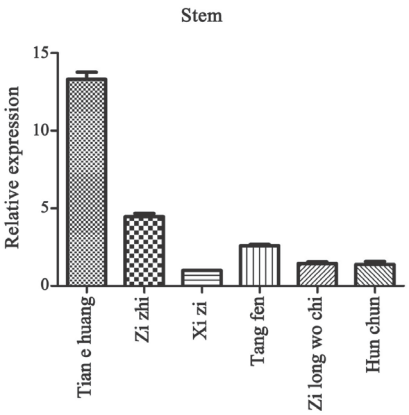

Figure 7. Relative expression of *RrTTG*1 in stems.

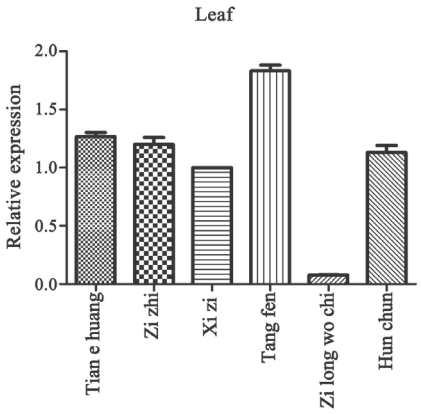

Figure 8. Relative expression of *RrTTG*1 in leafs.

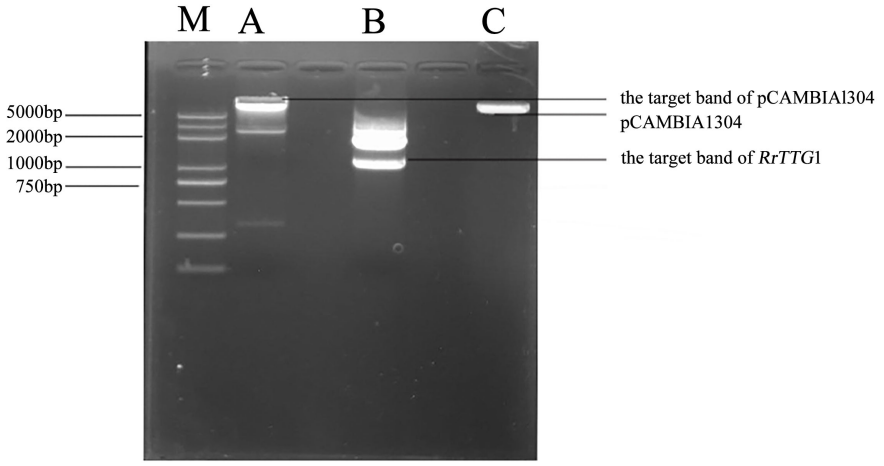

Figure 9. Identification of recombinant expression vector by double enzyme. M: Marker; A:pCAMBIA1304 by *Spe*I and *Pml*I double enzyme; B:*RrTTG*1 by *Spe*I and *Pml*I double enzyme; C: pCAMBIA1304 with no enzyme.

Through the NCBI homology alignment, the *RrTTG*1 amino acid sequence of *R. rugosa* has high homology with Fragaria vesca, Rubus chingii, Malus domestica, Nicotiana tabacum and other plants. In these plants, the function of *TTG*1 gene is different. In Malus domestica, *MaTTG*1 regulates the synthesis of anthocyanins [20]; In Nicotiana tabacum, the *NiTTG*1 gene is involved in the regulation of plant trichomes development [15]. The function of the *TTG*1 gene is diverse, and whether it is important in the formation of *R. rugosa* trichomes. The biological function remains to be verified by further research.

In several developmental and biochemical pathways of plants, *TTG*1 is involved in the regulation of trichomes, development of seed coat and seed coat pigment, biosynthesis of flavonoids and anthocyanidins [21]. In the *Arabidopsis thaliana ttg*1 mutant, the epidermis of stems and leafs and the subepidermal layer are completely deficient in anthocyanins, the seed coat cannot form anthocyanins and the outer seed coats appear as transparent traits, and trichomes function is absent without epidermis or less epidermis. The *ttg*1 mutant can cause so many changes in physiological processes, suggesting that *TTG*1 is a

gene ubiquitous in most regulatory pathways [10].

Generally, there are prickles on the stems of *R. rugosa*. The field morphology observation shows that there are more epidermis on the leaves and stems of *R. rugosa* "Zi long wo chi", *R. rugosa* "Xizi" and *R. rugosa* "Hun chun". There were fewer epidermis on the leaves and stems of the three varieties of *R. rugosa* "Tian e huang", *R. rugosa* "Zizhi" and *R. rugosa* "Tang fen". This study found that *RrTTG*1 gene was expressed in different *R. rugosa* varieties and different tissues, and the content in the leaves and stems of *R. rugosa* "Zi long wo chi", *R. rugosa* "Xizi" and *R. rugosa* "Hun chun". were lower. The contents of leaves and stems of *R. rugosa* "Tian e huang", *R. rugosa* "Zizhi" and *R. rugosa* "Tang fen" were higher. It is speculated that *RrTTG*1 gene may be associated with the formation of *R. rugosa* trichomes and may play a negative regulatory role in *R. rugosa* leaves and stems. In addition, the *RrTTG*1 gene has a large difference in the roots of six *R. rugosa* varieties. Except for the *R. rugosa* "Zi long wo chi", only a small amount of *RrTTG*1 was detected in the other five varieties. Whether *TTG*1 is a key gene, and whether there are other genes that control the development of *R. rugosa* trichomes remains to be further verified.

5. Conclusion

In conclusion, in this study, we cloned the *RrTTG*1 gene in *R. rugosa*, obtained the full-length cDNA of 1348 bp, and analyzed it by bioinformatics. The molecular formula $C_{1723}H_{2661}N_{465}O_{529}S_{12}$ was predicted, which was inferred to be unstable protein and Hydrophilic protein. Furthermore, it was concluded by fluorescence quantitative analysis that the *RrTTG*1 gene is involved in the regulation of the synthesis of *R. rugosa* epidermis, which is a negative regulation mechanism. This study is helpful for analyzing the formation mechanism of *R. rugosa* epidermis and provides some important information for breeding stingless *Rosaceae* family plants.

Conflicts of Interest

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

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